Quantitative microbiological exposure assessment of *Bacillus cereus* in cooked-chilled foods

ir. Jeff Daelman

UNIVERSITEIT GENT

FACULTY OF **BIOSCIENCE ENGINEERING**

Voor Katrien...

Promotors: **prof. dr. ir. Mieke Uyttendaele**
prof. dr. ir. Frank Devlieghere
prof. dr. ir. Liesbeth Jacxsens
Laboratory of Food Microbiology and Food Preservation
Department of Food Safety and Food Quality
Faculty of Bioscience Engineering, Ghent University

Dean: **prof. dr. ir. Guido Van Huylenbroeck**

Rector: **prof. dr. Paul Van Cauwenberge**

Examination Committee: **prof. dr. Bernard De Baets** (UGent, Chairman)
prof. dr. ir. Tom Van De Wiele (UGent, Secretary)
prof. dr. ir. Ingmar Nopens (UGent),
dr. Jeanne-Marie Membré (INRA-Oniris, France)
dr. ir. Maarten Nauta (National Food Institute, Denmark)
dr. ir. Guido Bresseleers (Belgian Ready Meal Association)
prof. dr. Marc Heyndrickx (ILVO - UGent)

ir. Jeff Daelman

Quantitative microbiological exposure assessment of *Bacillus cereus* in cooked-chilled foods

Thesis submitted in fulfilment of the requirements
for the degree of Doctor (PhD) in Applied Biological Sciences

Titel van het doctoraat in het Nederlands:

Kwantitatieve microbiologische blootstellingsschatting van *Bacillus cereus* in koelverse kant-en-klare maaltijden

Illustration: ...

To refer to this thesis:
Daelman, J. 2013. Quantitative microbiological exposure assessment of *Bacillus cereus* in cooked-chilled foods. Thesis submitted in fulfilment of the requirements for the degree of doctor (PhD) in Applied Biological Sciences. Faculty of Bioscience Engineering, Ghent University.

ISBN number: 978-90-5989-646-8

The research was performed at the:

Laboratory of Food Microbiology and Food Preservation,
Faculty of Bioscience Engineering, UGent, Ghent, Belgium

Unité Mixte de Recherche Sécurité des Aliments et Microbiologie
INRA/Oniris, Nantes, France

Woord vooraf

*"Doctoreren is **ook** werken" was het antwoord van Mieke toen ik nogal domweg zei "de meeste van mijn kameraden willen niet doctoreren, ze willen gaan werken". En hoe meer mijn doctoraat vorderde, hoe meer ik besefte dat die stelling volledig klopt. Doctoreren is inderdaad werken, maar het is veel méér dan dat. Het is jezelf leren kennen: doorbijten als een experiment nog maar eens niet lukt, eens vloeken als een paper nog maar eens geweigerd wordt en zuchten als je de hoeveelheid "rode balpen" op de finale versie van je manuscript ziet. En toch zal ik het missen, de voldoening om in een hoop data plots die ene afwijking te vinden, het plezier van iets te kunnen doen dat nog nooit werd gedaan.*

Eerst en vooral wil ik mijn drie promotoren bedanken: Liesbeth, Mieke en Frank. Bedankt om mij deze kans te geven, het waren vier bijzonder boeiende, leerrijke en verhelderende jaren. Het was een plezier om met jullie samen te werken en de verschillende invalshoeken en meningen te combineren. Ik ben jullie ook zeer dankbaar voor de mogelijkheid die ik kreeg om wat van de wereld te zien: Pisa, Dublin, Nantes, Warschau, Milwaukee, Berlijn, Istanbul, Marseille en Parijs; het is niet iedereen gegund op vier jaar. Ook An Vermeulen mag hier niet ontbreken voor haar deskundige begeleiding bij het modelleren en valideren, en het beantwoorden van duizend-en-één vragen over predictieve microbiologie.

J'aimerais aussi remercier tous mes collègues Nantais pour deux mois très amusants, avec beaucoup de petites pauses-café, et pour me pardonner, en riant, toutes mes fautes contre la langue de Molière. Je veux particulièrement dire merci à Jeanne-Marie, parce-que sans elle cette thèse n'aurait jamais été aussi bon, surtout en ce qui concerne la mathématique.

Als ik nog één collega hier met naam wil vermelden, dan is het wel Elien. Zonder haar was dit werk waarschijnlijk slechts de helft zo lijvig geweest of pas in 2015 voltooid. Geen beter geschenk voor een verstrooide dr. in spe dan een laborante met gezond verstand en goed humeur, die altijd wist wat er moest gebeuren, zelfs als ik het vergeten was.

Ook al mijn thesisstudentes verdienen een plaats in dit lijstje. Nanou, Tine, Rebecca, Sarah en

Elke, bedankt voor een jaar lang inzet, dataverzameling en denkwerk. Eveneens dankjewel aan alle collega's, zowel boven als in "den bureau beneden", voor alle hulp in het labo, de lunchpauzes vol onnozelheid, de vrijdagnamiddagen vol verstrooiing en de occasionele gouden tip.

Bedankt ook aan al mijn vrienden (en zeker "De Boys") en vriendinnen voor vele avonden en weekendjes vol zever, plezier, 'onnozeliteiten' en vriendschap.

Ook mijn ouders mogen hier niet ontbreken, het is dankzij hun steun dat ik zover geraakt ben; wie had ooit gedacht dat het demonteren van stofzuigers en broodroosters op een dag zou leiden tot een doctoraat. Ook mijn interesse voor voeding werd met de paplepel ingegeven, met een diëtiste als moeder en tandarts (bijgenaamd 'agar') als vader, was ik voorbestemd voor de levensmiddelenmicrobiologie. Bovenal ben ik jullie dankbaar voor de vrijheid en het vertrouwen dat ik van jullie gekregen heb. Mijn twee zusjes hebben hier eveneens hun plaats verdiend, het is immers niet simpel samen te leven met een grote broer die altijd gelijk moet (en zal) hebben.

Blijft er nog één iemand over...

Katrien, mijn steun en toeverlaat, mijn ijs-voetjes als ik te warm heb, mijn "ma schatteke toch" hulp in het relativeren, vroeger mijn vriendin, nu mijn verloofde en binnenkort mijn vrouw. Dankjewel, voor het verhelpen van het zuchten en het blazen als het onderzoek niet vorderde en voor het luisteren naar een hyper-snel-pratende-overdreven-enthousiasteling als het dan plots wel lukte. Bedankt, om mij te kalmeren, te motiveren, te verzorgen en om mij op tijd in mijn bed te steken, zonder jou was het niet gelukt. "Le jour où quelqu'un vous aime il fait très beau. Je peux pas mieux dire, il fait très beau..."

Jeff Daelman
Gent, September 2013

> *"We live in a society exquisitely dependent on science and technology, in which hardly anyone knows anything about science and technology."*
>
> *Carl Sagan, "Why We Need To Understand Science", The Skeptical Inquirer Vol. 14, Issue 3, (Spring 1990)*

Table of contents

List of abbreviations

% FD	% fail dangerous
ACMSF	Advisory Committee on the Microbiological Safety of Food
AIC	Akaike's information criterium
ALOP	Appropriate Level Of Protection
ASC	Aerobic Spore Count
a_w	Water activity
$a_{w,min}$	Minimal water activity to enable growth
$a_{w,opt}$	Optimal water activity for growth
BC	*Bacillus cereus*
BHI	Brain Heart Infusion broth
CB	*Clostridium botulinum*
CCFH	Codex Comite on Food Hygiene
CFU	Colony forming units
CRL	(EU) Community Reference Laboratories
D_x-value	Time (min) needed for 1 log (90%) reduction at x°C
DNA	Deoxyribonucleic Acid
DoP	Degree of Protection
EFSA	European Food Safety Authority
EPS	Expanded Polystyrene
EU	European Union
F_0	Sterilisation value at 121.1°C (250°F)
FAO	Food and Agricultural Organisation (of the United Nations)
FASFC	Federal Agency for the Safety of the Food Chain (Dutch: FAVV)
FBE	Faculty of Bioscience Engineering
FBO	Food Business Operator
FF	Flanders Food
FSMS	Food Safety Management System
FSO	Food Safety Objective
GHP	Good Hygiene Practices
GMP	Good Manufacturing Practices
GNG	Growth / no-growth
HACCP	Hazard Analysis Critical Control Point
ICMSF	International Commission on Microbiological Specifications for Foods

INRA	Institut National de Recherche Agronomique (FR)
LAB	Lactic Acid Bacteria
LFMFP	Laboratory of Food Microbiology and Food Preservation
LHC	Latin Hyper Cube
LMO	*Listeria monocytogenes*
LoD	Limit of Detection
LoQ	Limit of Quantification
MAP	Modified Atmosphere Packaging
MPRM	Modular Process Risk model
MYP	Mannitol egg Yolk Polymyxin
OD	Optical density
OD_{zero}	Average D of the first measurements
P_{70}/ P_0	Pasteurisation value at 70°C
P_{90}	Pasteurisation value at 90°C
PC	Performance criterium
PO	Performance Objective
PrC	Process Criteria
PPS	Pepton Physiological solution
PRP	Prerequisite program
QMRA	Quantitative Microbiological Risk Assessment
QMEA	Quantitative Microbiological Exposure Assessment
REPFED	Refrigerated and Processed Food of Extended Durability
ROC	Receiver Operating characteristic
RTC	Ready To Cook
RTE	Ready To Eat
RTH	Ready To Heat
RTR	Ready To Reheat
SASP	Small Acid-soluble Spore Proteins
SC	Shwartz Criterion
sc.	Scenario number
SRC	Sulphite reducing Clostridia
T_0	Day of production
T_{EoS}	End of Shelf life
TPAC	Total Psychrotrophic Aerobic Count
TSA	Trypton Soy Agar
TSB	Trypton Soy Broth
TTG	Time to growth (time before growth probability is $\geq$10%)
UBD	'use by' date
WTO	World Trade Organisation
WHO	World Health Organisation (of the United Nations)
xD	x decimal reduction
Y&M	Yeasts and Moulds

Introduction and objectives

Introduction and objectives

Consumer habits are rapidly changing; more women are participating in the workforce; people experience more time pressure because of leisure activities (Bowers, 2000) and have a lack of experience in cooking (Gofton, 1995). This means that less consumers take the time to prepare a homemade meal every day (Costa *et al.*, 2007). Refrigerated Processed Foods of Extended Durability (REPFEDs) were designed to maximise consumer convenience. They are also known as cooked-chilled foods, ready-meals, ready-to-eat meals, microwave-dinners. Since the mid 1990s, the European market for these products has increased, from €9.1 billion in 1996 to an estimated €25 billion in 2009 (Del Torre *et al.*, 2004; Business Insights, 2006).

These products thrive on the consumer's demand for convenience, but like all food products they should also be tasty and safe. Currently the microbial safety of these products is primarily assured using safe harbour heat treatments (dutch: *pasteurisatie barema's*), cold storage, packaging and product formulation. These safe harbour heat treatments originated in the 80's and 90's and have an excellent food safety record. However, they are based on older data, include a considerable safety margin (Gaze *et al.* 1989; ACMSF, 1992) and have a negative impact on product quality. Since the development of these safe harbours, society has changed (globalisation, consumer preferences, etc.) and our technological abilities have increased (control of logistics and the cold chain, new packaging materials, etc.). At the same time new food safety management concepts were implemented, which focus on the whole food chain (e.g. Prerequisite programs and HACCP) and our knowledge of the behaviour of microorganisms has increased. All these changes warrant a revision of these safe harbours and it is only natural for the industry to move towards milder heat treatments, but this cannot be done by compromising on food safety.

In REPFEDs that are in-pack-pasteurised, there are two microorganisms of concern: *Bacillus cereus* and *Clostridium botulinum*. Both are spore-forming, resistant to pasteurisation treatments, include cold-growing strains and are toxin producing pathogenic microorganisms. As a spore they can survive the pasteurisation and as a vegetative cell they are able to grow (slowly) at refrigerator temperatures. Because of this combination of characteristics, they are the primary microbial hazards in REPFEDs. If producers want to deviate from the safe harbour heat treatments, they should make sure that the consumer risk from exposures to these foodborne pathogens does not increase.

In this PhD the focus is on *B. cereus*, because it is generally more heat resistant than *C. botulinum*, because it can grow both anaerobically and aerobically and because it is easier to handle and study in a lab environment (less hazardous than *C. botulinum*). The primary objective of this PhD was to investigate the consumer exposure to *B. cereus* due to consumption of **industrially produced REPFEDs,** to identify critical points in the production process, to suggest potential risk mitigation strategies and to determine which prerequisites of product and process characteristics and storage conditions have to be respected in order to be able to deviate from the safe harbour heat treatments, without jeopardising food safety or increasing the consumer risk. The scope of this PhD is limited to REPFEDs that are industrially produced and distributed via supermarkets, REPFEDs distributed via small retailers (e.g. butchers) and caterers are not included. A schematic overview of the contents of this PhD is provided in Figure 1.

Objectives

1. To gain insight in the current REPFED production processes and different types of industrially produced REPFEDs on the market in Belgium. To collect factual information about the processing conditions (time, temperature, methods, etc.) and the current microbial contamination of REPFEDs during production and distribution.

2. To identify and solve data gaps in the current knowledge about *B. cereus* prevalence and behaviour in the relevant steps of the REPFED production and distribution process.

3. To determine the current consumer exposure to *B. cereus* from REPFEDs and to identify the critical steps in the production process and product shelf life with respect to consumer exposure to *B. cereus*

4. To evaluate potential risk mitigation strategies for reducing consumer exposure and to determine, under which conditions it is possible to deviate from the currently accepted safe harbour processes, without increasing consumer exposure for *B. cereus*.

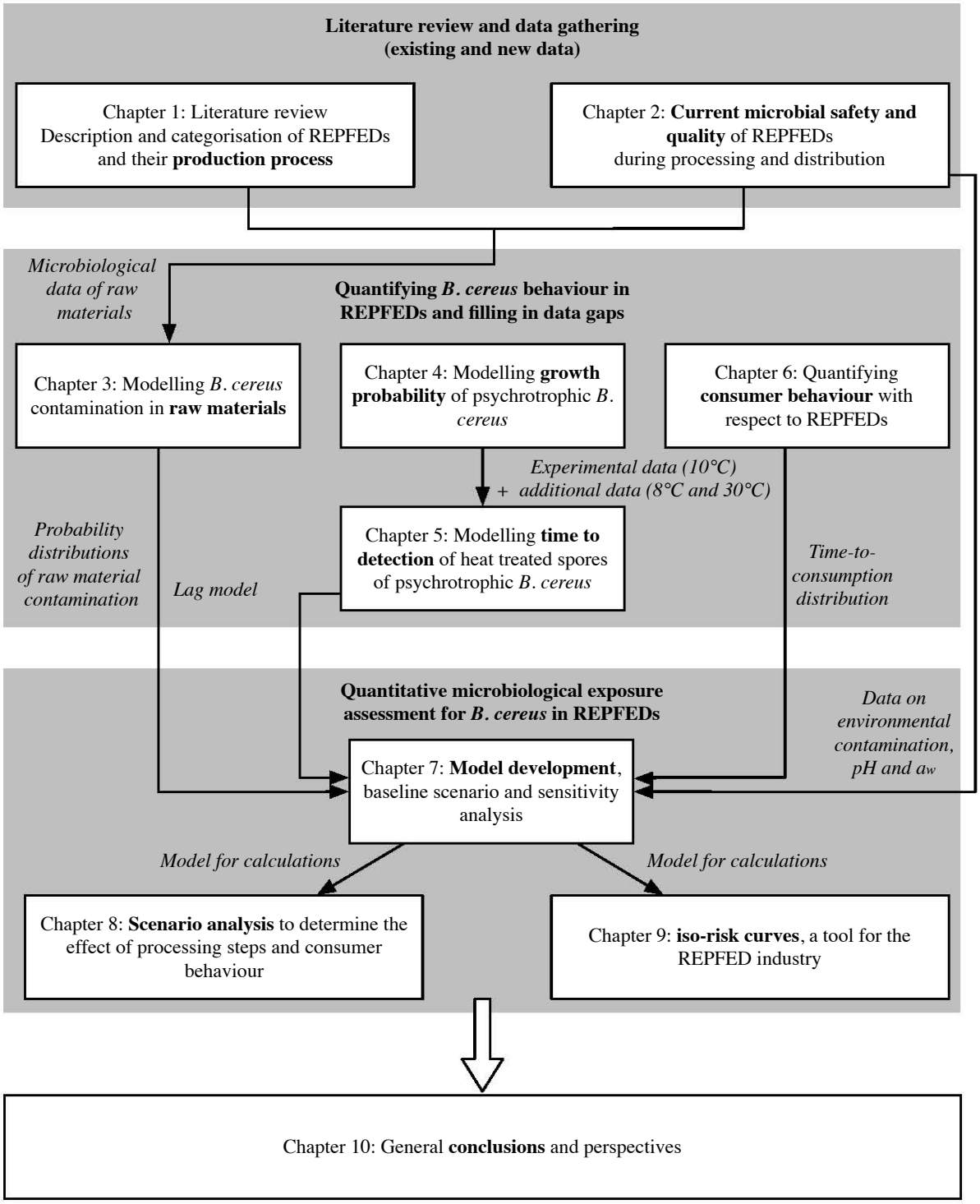


Figure 1: Outline of this PhD thesis, entitled: "Quantitative Microbiological Exposure Assessment of *Bacillus cereus* in cooked-chilled foods"

Summary - Samenvatting

Summary

Cooked-chilled foods, ready-meals, microwave dinners and **refrigerated and processed foods of extended durability** (REPFEDs), are all synonyms for a growing group of complex food products. Designed to maximise **consumer convenience**, these products have to be tasty, good-looking, easy to prepare and (above all) safe. To date, the microbial safety of these products is assured using a combination of product formulation (a_w, pH), pasteurisation, packaging (modified atmosphere, vacuum or air) and cold storage. The most common pasteurisation treatment applied to REPFEDs is **90°C for 10 min** or equivalent, a process designed to ensure a 6 log reduction of psychrotrophic (non-proteolytic) ***Clostridium botulinum*** strains. In contrast to its wide use, this time-temperature combination is based on the $D_{90°C}$-value (1.1 min) of a single *C. botulinum* strain isolated from cod and includes a three minute safety margin for variations in strain heat resistance (1.1 min $\times$ 6 +3 min $\approx$ 10 min).

Because of the effect of heat treatment on taste, texture and nutritional value, there is a trend towards (more) minimal processing and more natural foods. For REPFEDs this means avoiding preservatives ('clean label') and reducing the heat treatment intensity. However, both should be done without compromising consumer safety. In the case of REPFEDs there are three bacteria of concern: psychrotrophic *C. botulinum*, *Bacillus cereus* and *Listeria monocytogenes*. In this PhD the focus lies on *B. cereus* because it is the most **heat-resistant** of the three, because it can grow at low temperatures, and because it is **ubiquitous** in the environment and in REPFEDs. Although the **symptoms** are usually **mild** (diarrhoea or vomiting), several fatal cases of *B. cereus* food poisoning have been reported, of which two in Belgium.

The aim of this PhD is to evaluate the consumer exposure to *B. cereus* from REPFEDs and identifying the key factors during production and shelf life. To this end a **Quantitative Microbiological Exposure Assessment (QMEA)** is developed. Because REPFEDs are such a complex group, a segmentation is necessary. By visiting REPFED production sites and discussions with REPFED producers, **three different production processes** could be distinguished. The production processes and their characteristics are discussed in **chapter 1**. In addition to different production processes, the products also differ with respect to the **reheating advise given on the product label**. Based on this aspect a secondary classification is proposed to distinguish ready-to-eat foods from **ready-to-cook/heat/reheat foods**. Chapter 1 also focusses on the effects of

heat treatment, safe harbours, various predictive models and consumer behaviour on *B. cereus* and on the risk associated with REPFEDs.

In **chapter 2** the **current microbial quality and safety** of REPFEDs was given a closer look. A first assessment was made using existing microbiological data about the end-products. To gain more information about the microbial contamination during production a microbiological sampling of raw materials, half fabricates, contact materials and end products occurred during the **production process** of REPFEDs at five companies. In addition the recommended **heat treatment at consumer level** was simulated and quantified. The assessment and the existing data showed that the overall **microbial quality and safety of REPFEDs is acceptable**, but that raw materials and the production environment are a potential source of *B. cereus* (and *L. monocytogenes*). The simulated reheating at consumer level proved to be highly variable in temperature and P-value, both between products and within products.

In **chapter 3** a model is developed using the data collected in chapters 1 and 2 to estimate the **raw material contamination**. This model was based on the samples taken in chapter 2 and allows the input of a crude recipe (e.g. x% herbs, y% starch, ...) and is the first module of the final exposure assessment model for *B. cereus* in chapter 7.

In **chapter 4 and 5** a set of predictive **models for the growth probability and the lag time of heat treated *B. cereus* spores** was developed. This was necessary, because none of the existing predictive models was applicable to heat treated spores under cold storage. The available models either used vegetative cells, predicted inactivation or did not predict growth under cold storage. The developed models showed that pasteurisation or product formulation (a_w, pH) on their own, are not enough to prevent growth of *B. cereus*. Lower pH (<5.8) in combination with mild heat treatment is specifically efficient at prolonging the lag time and thus the shelf life. The model in chapter 5 enables the prediction of lag (as time to detection of growth) in function of product a_w, pH, storage temperature and pasteurisation time and temperature. The lag model is used in the final exposure assessment in chapter 7.

The **consumer behaviour** concerning home storage and consumption of REPFEDs is described in **chapter 6**. A questionnaire was completed by 874 respondents. The results showed that ±75% of the sample population consumed REPFEDs, but that only ±50% of the REPFED-consumers respects the shelf life or the reheating guidelines. More importantly the results of the questionnaire made it possible to determine a distribution of the **time between purchase and consump-**

tion. During development of the QMEA this variable had shown to be crucial, especially when the expected temperature abuse in a consumer refrigerator is taken into account. The results showed that most REPFEDs are consumed within the **first few days after purchasing**: 50% in two days and 90% in 7 days.

In **chapter 7** the development of the **quantitative microbiological exposure assessment model** is explained as well as the sensitivity analysis, model validation based on literature data and the results of the baseline scenario. Different **scenarios** are compared and discussed in **chapter 8**. In **chapter 9**, a set of iso-exposure curves (combinations of a_w, pH, shelf life and heat treatment yielding the same exposures) are presented together with a set of risk-boundaries for *B. cereus* and *C. botulinum*. Sensitivity and scenario analysis showed that raw material contamination, hygiene during processing and consumer behaviour are of the greatest importance to assure microbial food safety. The iso-risk curves illustrate the potential use of the QMEA for product innovation support. It also showed that the risk-boundaries for *B. cereus* are larger than those of *C. botulinum* (i.e. *B. cereus* requires more pasteurisation or a lower pH to prevent growth, compared to *C. botulinum*). The general conclusions of this PhD and perspectives for future research are discussed in **chapter 10**.

Samenvatting

Kant-en-klare maaltijden, **koelverse maaltijden** of bereide gerechten, het zijn allemaal synoniemen voor een groeiende groep van complexe levensmiddelen die speciaal ontworpen zijn met het oog op **consumentencomfort**. De producten moeten goed smaken, er goed uitzien, gemakkelijk te bereiden zijn en (bovenal) veilig zijn. Tot op heden wordt de microbiële veiligheid van deze producten verzekerd door een combinatie van product formulering (a_w, pH), pasteurisatie, verpakking en gekoelde bewaring. Het meest voorkomende pasteurisatie barema is **90°C gedurende 10 minuten**. Een proces dat ontworpen is om een 6 log reductie te veroorzaken van psychotrope (niet-proteolytische) ***Clostridium botulinum*** stammen. Deze tijd-temperatuur combinatie is gebaseerd op de $D_{90°C}$-waarde (1.1 min) van één *C. botulinum* stam afkomstig uit kabeljauw. Bovendien bevat dit barema een veiligheidsmarge van 3 minuten om te compenseren voor variabiliteit in hitteresistentie tussen verschillende stammen (1.1 min $\times$ 6 +3min $\approx$ 10 min).

Omdat hittebehandelingen ook de smaak, textuur en voedingswaarde van een product beïnvloeden is er een trend naar (meer) minimaal bewerkte en meer natuurlijke levensmiddelen. Voor koelverse maaltijden betekent dit het vermijden van bewaarmiddelen ('*clean label*'), het verkorten van de pasteurisatie tijd en het verlagen van de pasteurisatietemperatuur. Deze aanpassingen mogen echter niet ten koste gaan van de voedselveiligheid. Voor koelverse maaltijden zijn er drie relevante micro-organismen: psychrotrofe *C. botulinum*, *Bacillus cereus* en *Listeria monocytogenes*. In dit doctoraat ligt de focus op *B. cereus*, omdat dit micro-organisme het meest **hittebestendig** is van de drie, kan groeien bij koelkast temperaturen, **alomtegenwoordig** is in het milieu en hoewel de **symptomen meestal mild** zijn (overgeven en diarree) zijn er toch fatale gevallen van *B. cereus* voedsel-intoxicaties bekend, waaronder twee in België.

Het doel van dit doctoraat is het evalueren van de consumentenblootstelling aan *B. cereus* via koelverse maaltijden en het bepalen van belangrijkste factoren tijdens productie en houdbaarheid van deze producten. Om deze doelen te bereiken werd een **kwantitatieve microbiologische blootstellingsschatting** (Engels: *Quantitative Microbiological Exposure Asessment* of QMEA) uitgevoerd. Omdat koelverse maaltijden een complexe groep zijn, was een opdeling van het gamma nodig. Tijdens verscheidene bezoeken aan productie-sites en discussies met producenten werden op basis van het productie proces **drie verschillende types** gedefinieerd naargelang het productieproces. Het productieproces en de karakteristieken van deze drie types wordt toegelicht in **hoofdstuk 1**. Naast een verschil in productieproces, verschillen de pro-

ducten ook in de **opwarming die wordt aangeraden op de verpakking.** Op basis van deze eigenschap werd een **tweede classificatie** voorgesteld. Verder omvat hoofdstuk 1 ook meer informatie over de drie organismen, pasteurisatie barema's, predictieve microbiologische modellen en consumentengedrag.

In **Hoofdstuk 2** wordt de **huidige microbiële kwaliteit en veiligheid** van koelverse maaltijden belicht. Een eerste inschatting hiervan werd gemaakt op basis van bestaande analyseresultaten van de eindproducten. Om meer informatie te verwerven over de microbiële contaminatie doorheen het productieproces, werd een **uitgebreide staalname van het productieproces** gedaan bij vijf producenten van koelverse maaltijden. Daarnaast werd ook de **hittebehandeling door de consument gesimuleerd en gekwantificeerd**. Zowel de bestaande als de nieuwe analyse resultaten toonden aan dat de **huidige microbiële kwaliteit en veiligheid acceptabel is**, maar dat grondstoffen en de productie-omgeving een potentiële bron van *B. cereus* en *L. monocytogenes* zijn. De resultaten van de gesimuleerde opwarming door de consument waren zeer variabel in zowel temperatuur als pasteurisatiewaarde, en dit zowel tussen verschillende producten of meerdere herhalingen van hetzelfde product.

In **hoofdstuk 3** wordt een model voorgesteld voor het inschatten van de ***B. cereus* contaminatie op grondstoffen**. Dit model is gebaseerd op de data uit hoofdstuk 2 en laat toe om een 'grof' recept in te voeren (b.v.: x% kruiden en specerijen, y% zetmeel-componenten, ...). Het is de eerste module in de finale blootstellingsschatting in hoofdstuk 7.

In **hoofdstuk 4 en 5** worden een aantal modellen voor **hittebehandelde *B. cereus* sporen** voorgesteld. De ontwikkeling van deze modellen was nodig, omdat geen van de bestaande modellen zowel sporen, pasteurisatie en bewaring bij lage temperatuur omvat. De modellen toonden aan dat pasteurisatie of product formulering (a_w, pH) op zichzelf onvoldoende zijn om groei van *B. cereus* te voorkomen. Een verlaagde pH (<5.8) in combinatie met een milde hittebehandeling is echter zeer efficiënt in het vertragen van de uitgroei van *B. cereus*. Het model in hoofdstuk 5 laat toe om de lag-tijd (als tijd tot detectie) te voorspellen in functie van a_w, pH, hittebehandeling en bewaartemperatuur en werd gebruikt als lag-model in de finale blootstellingsschatting.

Het **gedrag van consumenten** met betrekking tot koelverse maaltijden wordt beschreven in **hoofdstuk 6**. Een enquête werd afgenomen bij 874 personen. Daaruit bleek dat ± 75% van de bevolking koelverse maaltijden consumeert, maar dat slechts 50% de houdbaarheidsdatum of de richtlijnen voor opwarming respecteert. De data uit de enquête werd gebruikt om de distributie

te bepalen van de **tijd tussen aankoop en consumptie** van het product. Tijdens de ontwikkeling van de blootstellingsschatting was deze variabele namelijk als cruciale factor aan het licht gekomen, vooral omdat ook de temperatuurvariatie in de consumenten koelkast mee in rekening werd gebracht. De resultaten toonden aan dat de meeste koelverse maaltijden **worden geconsumeerd in de eerste paar dagen na aankoop**: 50% in de eerste twee dagen, 90% in de eerste 7 dagen.

In **hoofdstuk 7** wordt de ontwikkeling van het **kwantitatieve microbiologische model voor blootstellingsschatting** beschreven, alsook de sensitiviteits-analyse, modelvalidatie gebaseerd op literatuur en de resultaten van het basis scenario. Verschillende **scenario's** worden vergeleken en besproken in **hoofdstuk 8** en in **hoofdstuk 9** wordt een set iso-risicocurves en risicogrenzen (of randvoorwaarden) voorgesteld. Sensitiviteits- en scenario-analyse toonden aan dat contaminatie van grondstoffen, hygiëne tijdens processing en consumentengedrag van het grootste belang zijn voor de microbiologische veiligheid van koelverse maaltijden. De iso-risicocurves tonen het potentieel van kwantitatieve blootstellingsschatting voor de ondersteuning van productinnovatie. Ze tonen daarnaast ook dat de randvoorwaarden voor *B. cereus* strikter zijn dan voor *C. botulinum* (i.e. om *B. cereus* groei te voorkomen, is een intensere hittebehandeling en/of lagere pH nodig dan voor *C. botulinum*). De algemene conclusies van dit doctoraat en mogelijke pistes voor verder onderzoek worden besproken in **hoofdstuk 10**.

Chapter 1

Literature review

Partially redrafted after:

Daelman, J., Jacxsens, L., Devlieghere, F. & Uyttendaele, M. (2013b). Microbial safety and quality of various types of cooked chilled foods. *Food Control*, 30(2):510–517

1.1 Refrigerated Processed Foods of Extended Durability

Refrigerated Processed Foods of Extended Durability (REPFEDs), also known as cooked-chilled foods or ready-meals, ready-to-eat meals, microwave-dinners are a diverse group of food products, which are designed to resemble a 'home-made' meal as close as possible. It is not possible to give a definition based on recipe, appearance, ingredients, origin etc. There is an endless number of possible recipes/dishes. For an illustration of this diversity consult table 2.4 in chapter 2 (p.59).

The fact that REPFEDs are difficult to define, is visible in the lengthy definition given by the Codex Committee on Food Hygiene (CCFH) (1999). They use the name "refrigerated packaged foods with extended shelf life" and define them as:

> "*... low-acid refrigerated foods that are* ***heat treated*** *and are* ***susceptible to outgrowth of pathogenic microorganisms*** *during their extended shelf-life.*
>
> *The foods which the provisions of this code addresses are products that:*
>
> - *are intended to be* ***refrigerated*** *during their shelf life to retard or prevent the proliferation of undesirable microorganisms;*
> - *have an* ***extended shelf life*** *of more than 5 days;*
> - *are* ***heat treated*** *or processed using other treatments to reduce their original microbiological population;*
> - *are low acid, that is, with* ***pH > 4.6*** *and have high* ***water activity (a_w) > 0.92****;*
> - *may use* ***hurdles*** *in addition to heat or other treatments and refrigeration, to retard or prevent the proliferation of undesirable microorganisms;*
> - *are* ***packaged****, not necessarily hermetically,* ***before or after processing*** *(heat or other preservation treatments);*
> - *may or may not require* ***heating prior to consumption****.*
>
> ***Examples*** *of such products are:*
>
> - ...
> - *cooked refrigerated ready to eat meats, poultry, seafood and their products, sauces, dips, vegetables, soups, egg products, pasta, ...*

> *This Code **excludes**: raw foods, frozen foods, low acid canned foods, acid and acidified foods stored at ambient temperature, smoked fish, . . . "*
> (CCFH, 1999)

While this definition is both accurate and comprehensive, the acronym REPFED will be used to emphasise that the products under discussion in this thesis are not only packaged but also pasteurised.

1.1.1 General description

The common denominator for all REPFEDs is the production process (Figure 1.1), which is characterised by two heating steps. In the first part of the production process the components of the REPFED are prepared (cut, minced, cooked, baked,. . .) like a 'home-made' meal, only in larger quantities (ca.100 - 1000kg). Once the component has been prepared it is divided in to smaller portions (ca. 100 - 500g). In the second part of the production process, the different components are put together (i.e. assembled or mixed) to form the final product (ca. 400 - 1500g). These assembled products are then packaged and pasteurised. In some cases the preparation process is cold and the cooking is integrated in the pasteurisation. This is usually done for recipes with a long cooking time (e.g. stew, ragout . . .). Because REPFEDs are not sterilised but pasteurised (60-95 °C for several minutes), various microorganisms are able to survive the heat treatment and cold storage is therefore vital to assure microbial safety and quality of the product during shelf life. It is precisely the combination of these two steps, pasteurisation and cold storage, that determine a REPFED.

Because REPFEDs are designed to fulfil the demand for safe, convenient foods of good sensorial quality, the manufacturer has to rely on a combination of mild heat treatment (i.e. pasteurisation), appropriate product formulation (a_w, pH, preservatives, etc.), packaging (MAP, vacuum, air), cold storage and shelf life limitation to assure the safety of these food products (Del Torre *et al.*, 2004). The combination of multiple parameters is beneficial for product quality as each stress factor can be less stringent compared to when it would be applied alone, a concept known as combination or hurdle technology (Leistner & Gorris, 1995).

REPFEDs are a growing market, over the last decade the European turnover of these food products increased by more than 16%, from €9.1 billion in 1996 to €10.6 billion in 2005 (Del Torre *et al.*, 2004; Membré *et al.*, 2009). The UK market for chilled foods went from £7.8 billion in 2007 to an estimated £8.7 billion in 2010, which constitutes an increase of 11.5%

in four years. The combined EU-US market for these products has steadily grown from $29.2 billion in 2005 to $32.6 billion in 2009. A growth that is likely driven by the consumer's sense of 'lack of time' (Business Insights, 2006).

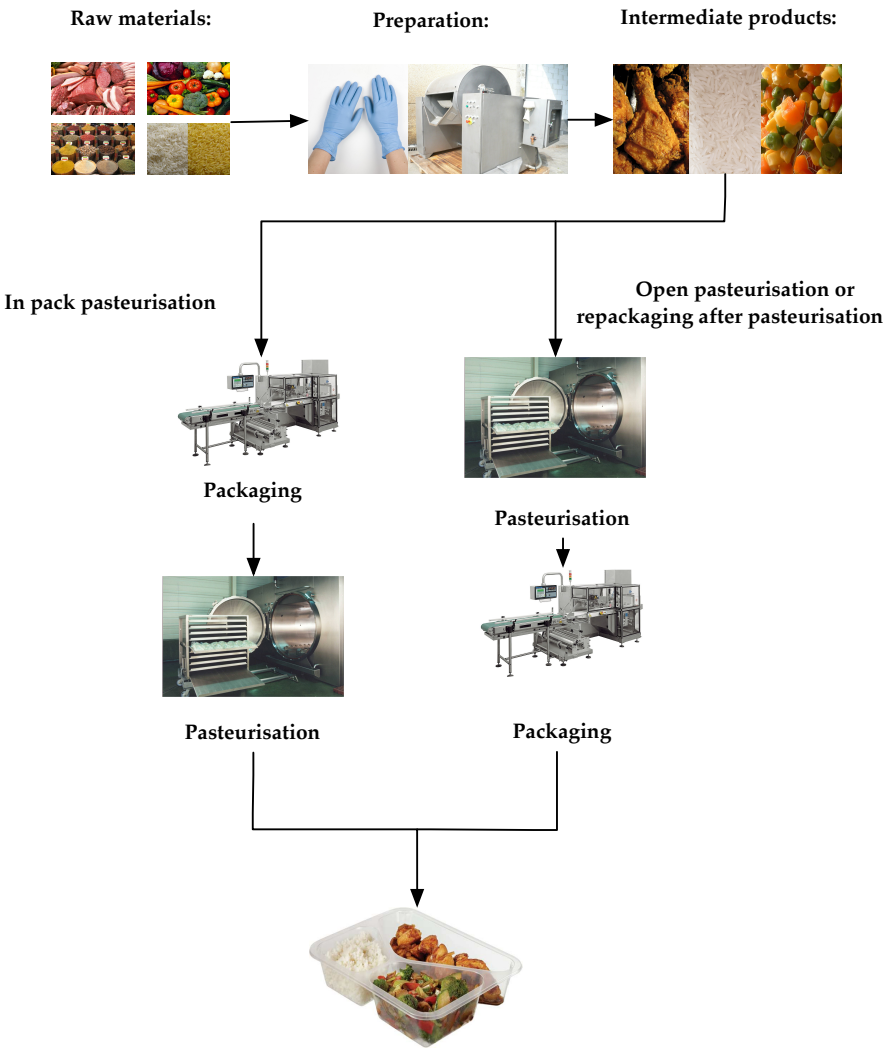


Figure 1.1: Simplified graphic representation of the REPFED production process

1.1.2 Classification of REPFEDs according to their production process

Figure 1.1 illustrates that packaging can take place before or after pasteurisation, a choice that significantly affects the possible risk of the product. Information on the characteristics of REPFEDs and on the differences in production processes was obtained from five REPFED-producing com-

panies. This information included a list of specifications and process flow parameters (e.g. time-temperature combinations during heating) as well as information on the microbial safety of the end products and the preparation instructions for the consumer. Based on this information a generic flowchart was developed for the different types of production process.

REPFEDs were classified according to the production process they received. Three types of generic production processes were identified, each with a different pasteurisation value applied during heat treatment or with a different potential for post-process contamination. The respective flowcharts are shown in Figure 1.2.

Type 1 products are subjected to an in-pack-pasteurisation for at least 10 min at 90°C or equivalent ($P_{90} \geq 10$ min). This heat treatment is generally recognised as safe harbour and will eliminate vegetative cells of *Listeria monocytogenes*, *Bacillus cereus* and *Clostridium botulinum*. Heat-resistant spores of *B. cereus*, *C. botulinum* and other spore forming microorganisms can survive this treatment, but they are expected to be sublethally injured (Membré *et al.*, 2009).

Type 2 products are subjected to an in-pack-pasteurisation of at least 2 min at 70°C or equivalent (P_{70} or $P_0 \geq 2$ min). This heat treatment is designed to result in at least a 6D reduction of *L. monocytogenes*, but spores of *B. cereus* and *C. botulinum* are able to survive this treatment. Due to the milder heat treatment, the injury to the spores is expected to be less extensive compared to type 1 products (Membré *et al.*, 2009).

Type 3 products are either pasteurised in an open-pack or pasteurised in pack and repackaged after pasteurisation. This type of products are not defined by a single P-value, both processes mentioned above ($P_{90} = 10$ and $P_{70} = 2$) are possible. This open-pasteurisation or repackaging introduces the risk of post-process contamination with *L. monocytogenes* or other zoonotic enteric pathogens, which can be present in the production environment (e.g. ceilings, food contact materials, gloves) or in ingredients that are added after heat treatment (e.g. meat cubes, grated cheese) (Reij *et al.*, 2004). Due to this potential for recontamination, type 3 products are a particular risk with respect to *L. monocytogenes*.

From the flowchart (Figure 1.2) it is clear that the production process also includes a second thermal preparation processes (e.g. cooking, blanching, baking), which precedes the packaging and pasteurisation process. These heat treatment processes are not taken into account to assure food safety, since they are designed to sensorially or technologically prepare the products and are not validated for delivering a reduction in microbial contamination. However, the temperatures obtained during these processes (60-100°C) are sufficiently high to reduce, to some extent, the

microbial contamination of a product (Gaze, 2006). Consequently, these processes also contribute to the inactivation of the microbial flora.

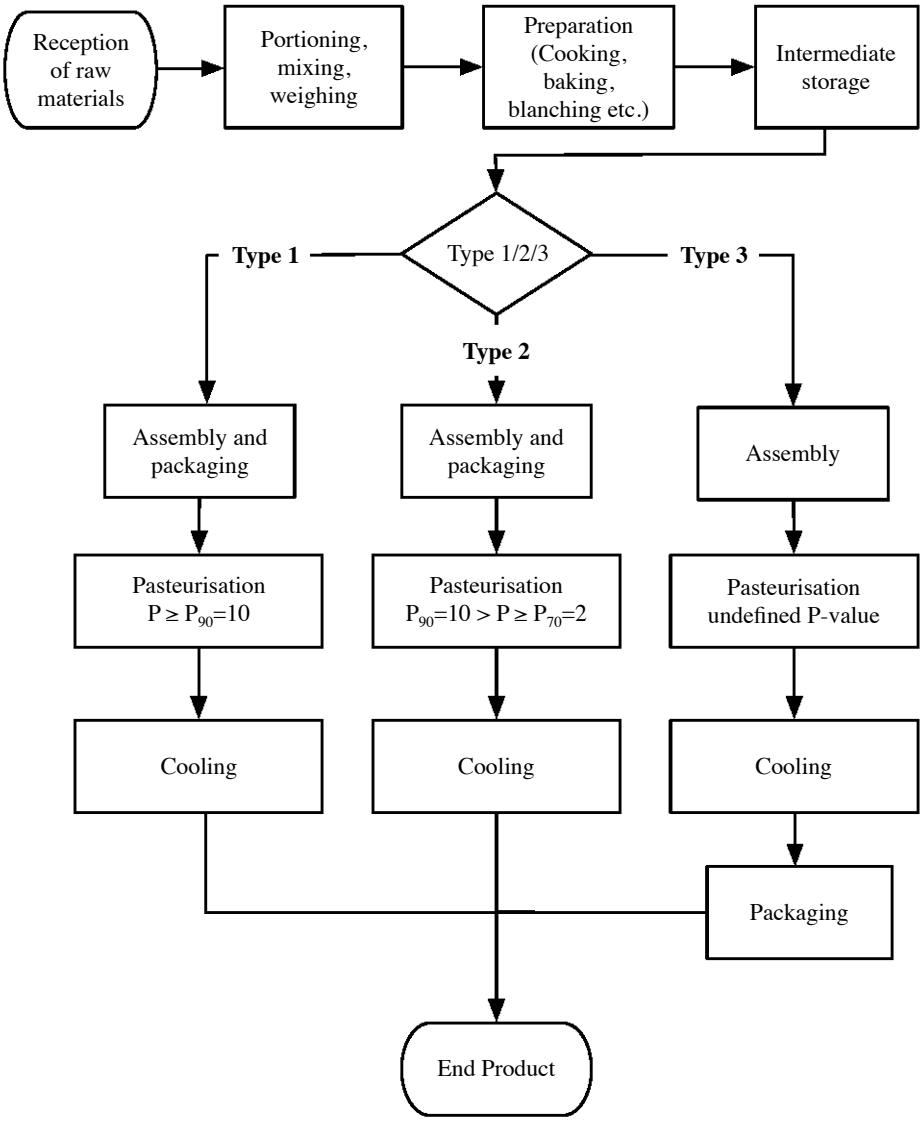


Figure 1.2: Flowchart classifying REPFEDs according to the production process. Type 1: Cooked-chilled foods (P-value ≥ P_{90}=10), Type 2: Post-process pasteurisation (P_{70}=2 ≥ P-value < P_{90}= 10), type 3: Open pack pasteurisation or pasteurisation in pack with repackaging after pasteurisation.

1.1.3 Classification of REPFEDs according to consumer behaviour

Reheating of REPFEDs at consumer level is mostly done for sensorial reasons, but may inactivate or damage microorganisms that are present in the food product. Therefore, the intensity of the heat treatment will also influence the exposure of consumers to certain pathogenic microorganisms (Figure 1.3). If the heat treatment is validated to achieve a 6D reduction of *L. monocytogenes* ($P_{70} \geq 2$ min) the product is classified as a ready-to-heat (RTH) product. When applied correctly by the consumer, this treatment should eliminate any *L. monocytogenes* present due to recontamination and/or outgrowth. However, some products do not achieve a heat treatment equivalent to two minutes at 70°C ($P_{70} < 2$ min), which means there is no extra hurdle for microorganisms to overcome but only a sensorial preparation of the product. These products are classified as ready-to-reheat (RTR) products. A third category of REPFED products is not heated prior to consumption; these are classified as ready-to-eat (RTE) products. While both the RTR and RTE products are a risk group with respect to *L. monocytogenes*, the RTH group is not, given that the consumer respects the recommended heat treatment. As manufacturer, validation and clear communication of this heat treatment is important, but the effectivenes of the final reheating depends on the consumer (Clayton & Griffith, 2003).

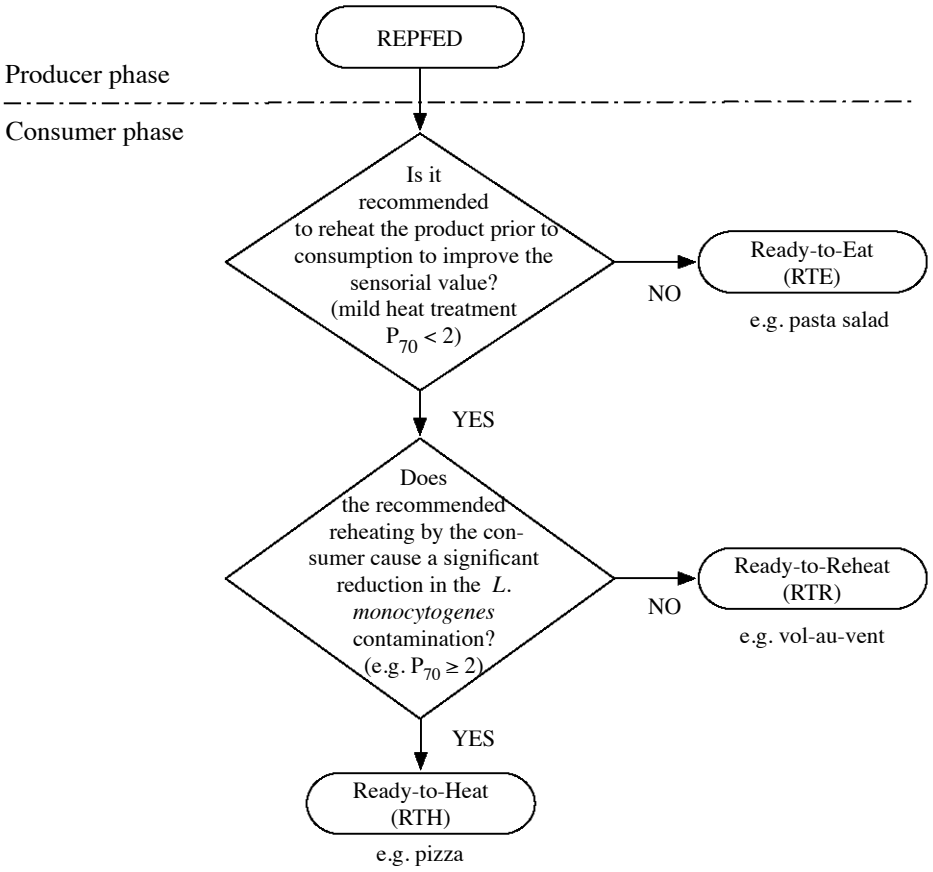


Figure 1.3: Flowchart classifying REPFEDs according to the reheating recommended to the consumer. Note that examples illustrate typical foods, but actual categorisation will depend upon the time-temperature combinations recommended and validated by the producer.

1.2 Pathogens of concern

As stated earlier, there are three pathogens of concern in REPFEDs: *B. cereus*, *C. botulinum* and *L. monocytogenes* (Carlin *et al.*, 2000a; Mossel & Struijk, 1991). Interestingly, Kennedy *et al.* (2005) reported that of the 1,020 Irish households participating in a survey on food safety, most respondents had heard of *Salmonella* (92.9%) and *Escherechia coli* O157 (77%), but that only 45.2% had heard of *L. monocytogenes* and that *B. cereus* or *C. botulinum* are even less known (<20%).

1.2.1 *Bacillus cereus*

1.2.1.1 General description

B. cereus are large (>0.9μm), motile, Gram positive, facultative anaerobic, rod-shaped pathogenic bacteri. It is part of the *B. cereus* group, along with *Bacillus anthracis* (anthrax), *Bacillus thuringiensis* (Bt Cotton), *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus weihenstephanensis*. Nearly all of these species comprise both pathogenic and harmless strains. Pathogenicity is related to the production of toxins, either emetic toxin (cereulide) or diarrhoeal toxins (Nhe or Hbl). Although most *B. cereus* strains hold one or more toxin genes, their pathogenicity is variable. The clinical *B. cereus* strains do not seem to possess specific combinations of genes or polymorphisms, but just a higher enterotoxin expression (Guinebretière *et al.*, 2002). In addition, some species outside the group have also been reported to produce the toxin (From *et al.*, 2005).

B. cereus is ubiquitous in the environment, mostly in soil and it is therefore virtually impossible to prevent the presence of *B. cereus* in raw materials. *B. cereus* is a very diverse species, the most important difference between strains is the ability to grow at low temperatures. Luckily, not all strains of *B. cereus* are psychrotrophic and even those that are, have different minimal growth temperatures. Samapundo *et al.* (2011b) tested the minimal growth temperature of 380 *B. cereus* strains isolated from cooked-chilled foods and cooked-chilled food ingredients. Only 2.6% was able to grow at ≤7°C, and only 6.2% at 8°C. At 9°C and 10°C the percentage of strains able to grow was significantly higher: 49.7%, 87.9% respectively. While these psychrotrophic strains are able to grow at low temperatures, they are usually more heat sensitive than their mesophilic counterparts reducing the probability that they survive the pasteurisation process (Table 1.1 and Carlin *et al.* (2000a)).

While *B. cereus* is reported to only sporulate in the presence of oxygen, it is commonly known as a facultative anaerobic microorganism (Ceuppens *et al.*, 2011; Gibbs, 2002). High concentrations of CO_2 have been reported to delay or prevent growth. Carlin *et al.* (2000a) reported that at 10°C, 50% CO_2 was sufficient to prevent growth of four (out of five) *B. cereus* strains. At the same time, these conditions were not able to prevent growth of spoilage *Bacillus* strains. Recent studies suggest that *B. cereus* is only able to grow anaerobically if temperature is sufficiently high. de Sarrau *et al.* (2012) demonstrated that anaerobic conditions and low temperatures (15°C) drastically reduced the growth rate and biomass production. At 12 °C, anaerobic growth was no longer possible. This might be due to the inability of *B. cereus* to produce sufficient branched chain fatty acids and unsaturated fatty acids under anaerobic conditions, which are necessary to

Table 1.1: Growth limits of mesophilic and psychrotrophic *B. cereus* (Dufrenne *et al.*, 1994; Gibbs, 2002; ICMSF, 1996; Fermanian *et al.*, 1994; Membré *et al.*, 2008; EFSA, 2005a)

Characteristic	mesophilic	psychrotrophic
Temperature growth range	10-55 °C	4-42 °C
Optimal growth temperature	30 - 37 °C	30 - 37°C
pH-range	4.3 - 9.3 (opt: 6-7)	
Minimum a_w for growth	0.912-0.950	
$D_{90°C}$[a]	4.8- > 200 min	4.6 -14 min
$D_{90°C}$[b]	1.5 - 104 min	0.9-19min
Heat stability of emetic toxin[c]	180 min at 100°C, or 30 min at 121°C, but only at pH >9	

[a] Dufrenne *et al.* (1994), [b] Membré *et al.* (2008), [c] Rajkovic *et al.* (2008)

maintain membrane fluidity at low temperatures (de Sarrau *et al.*, 2012). *B. cereus* has also been reported to form long filaments under anaerobic and cold conditions. These filaments increase the biomass and optical density of the growth medium, but are still counted as one CFU. When this filament is subsequently incubated at warmer temperatures, it splits into several daughter cells (de Sarrau *et al.*, 2013).

1.2.1.2 Two types of foodborne illnesses

The consumption of *B. cereus* can lead to two distinct types of foodborne gastrointestinal illnesses: the emetic syndrome and the diarrhoeal syndrome. The first syndrome is a food intoxication caused by the ingestion of an emetic (vomit inducing) toxin preformed in the food: cereulide (Agata *et al.*, 1995). This is an extremely stable dodecadepsipeptide that acts as a potassium ionophore, causing mitochondrial swelling (Mikkola *et al.*, 1999). While it is toxic to humans, it is likely to serve a purpose for the producing strains. Ekman *et al.* (2012) reported that cereulide might allow the producing strains to compete and grow better in a K^+ deficient environment. Cereulide is resistant to proteolysis, extreme pH and high temperature (Granum & Lund, 1997; Rajkovic *et al.*, 2008). The emetic syndrome is characterised by nausea, vomiting and sometimes also diarrhoea. The symptoms start 1-5 hours after ingestion and can last for 6-24 hours, complete recovery usually occurs within 24h (Gibbs, 2002; ICMSF, 1996). Production of the emetic toxin is reported to be higher at 12-15°C than at 20-30°C, but will require longer incubation periods. It is generally assumed that the range of environmental conditions that permits

cereulide production is narrower than the the range permitting *B. cereus* growth (Finlay *et al.*, 2000, 2002).

Only a certain percentage of *B. cereus* strains produces the emetic toxin (1-5%). But the emetic strains are isolated much more frequently from clinical samples (32.8%) (Carlin *et al.*, 2006; Altayar & Sutherland, 2006; Samapundo *et al.*, 2011c; Ceuppens *et al.*, 2011; Hoton *et al.*, 2009). Although psychrotrophic strains are not generally considered to produce the emetic toxin, some cases have been reported (Ehling-Schulz *et al.*, 2005; Altayar & Sutherland, 2006). In addition Thorsen *et al.* (2009) tested the cereulide production of two mesophilic *B. cereus* strains and noted that while the strains could not grow at 5 or 8°C, these initial temperatures did affect the behaviour of the strains when the temperatures were subsequently raised to 25°C. When the temperature during pre-incubation was lower (5 instead of 8°C), the production of cereulide during the simulated temperature abuse (25°C) was 9 to 40 times higher.

The second syndrome is the diarrhoeal syndrome. This is a food infection caused by the ingestion of viable *B. cereus* spores, which germinate in the gut and form enterotoxins. Three cytotoxins have been associated with diarrhoeal disease: haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe) and cytotoxin K (Arnesen *et al.*, 2008). Enterotoxins can also be preformed in the food, but because they are inactivated in the stomach, the concentration would need to be so high that the product would no longer be acceptable for human consumption (Granum, 1997). Of the two types, the emetic syndrome is probably the most dangerous, because of its association with life-threatening acute conditions such as liver failure, liver necrosis, rhabdomyolysis and necrosis of colon mucosa and submucosa (Mahler *et al.*, 1997; Naranjo *et al.*, 2011).

Generally, the emetic syndrome is mostly linked to starch containing products, while the diarrhoeal syndrome can occur in a wide variety of food products (Ceuppens *et al.*, 2011). Ankolekar & Labbé (2009) reported that diarrhoeal *B. cereus* strains were able to survive the cooking process of rice, but that growth after heat treatment was poor (at 20°C). However, under the same conditions, emetic strains would increase 20-fold.

In addition to causing foodborne diseases, *B. cereus* is also known to cause septicaemia, meningitis, gingival and occular infections (keratitis, endophthalmitis, and panophthalmitis) (Teyssou *et al.*, 1998; Kotiranta *et al.*, 2000). In contrast to its pathogenic potential, certain *B. cereus* variants are used as probiotic (Hoa *et al.*, 2000; Klein, 2011).

1.2.1.3 Prevalence and epidemiological association with REPFEDs

B. cereus is commonly found in a large variety of foods: spices, flour, bakery products, pasteurised milk, fresh vegetables, vegetable salads, milk powder and powdered instant formulae (EFSA, 2005a). The European Rapid Alert System for Food and Feed contains 109 notifications concerning *B. cereus* (Anonymous (2013) between 1983-2013). Most notifications are for herbs and spices, prepared dishes and snacks or for fruit and vegetable products.

B. cereus has also been widely reported in REPFEDs. Del Torre *et al.* (2001) sampled gnocchi, a potato based REPFED, and found that 33% of the unstored samples contained *B. cereus*, but counts were low for unstored and refrigerated samples (<10^2 CFU/g). When the product were stored under temperature abuse (12-20°C), *B. cereus* counts increased up to 10^5 CFU/g.Valero *et al.* (2002) tested fresh vegetables designated to be used as REPFED ingredients, as well as the corresponding REPFEDs. Prevalence on fresh vegetables ranged between 0% on garlic samples (n=6) and 14.2% for onions (n=7), to 90% for tomatoes (n=10) and even 100% for fresh peppers (n=11). Average counts ranged from <1 CFU/g to 10^4 CFU/g. According to the same study, the prevalence of *B. cereus* on the REPFEDs was between 0% (n=7) and 44.4% (n=9) and contamination was very low (< 3 - 8 MPN/g). Rajkovic *et al.* (2006) sampled vacuum packed potato puree and reported that 5 of the 6 final products that were sampled, contained *B. cereus* (<1 - 3.95 log CFU/g). Choma *et al.* (2000b) detected *B. cereus* in 20% of REPFEDs samples, but at concentrations less than 10 CFU/g. When the products were stored at room temperature for four to twelve days, 70% of products was positive for *B. cereus*. Carlin *et al.* (2000b), did not find any *B. cereus* in 42 samples of REPFEDs taken immediately after the production process. After storage at room temperature, 13 samples were positive. More recently, Samapundo *et al.* (2011b) sampled various food products (cooked pasta, lasagna, béchamel and Bolognese sauce, fresh minced beef, fresh-cut vegetables and raw basmati rice) and found that 56.3% (n=575) of the samples contained *B. cereus*. It should be clear from this (non-exhaustive) list, that *B. cereus* is a common microorganism in many food products and specifically in REPFEDs and REPFED-ingredients.

1.2.1.4 Cases and outbreaks worldwide

Data about *B. cereus* outbreaks and foodborne diseases is scattered. EFSA (2005a) reported very different percentages for different European countries. Ranging from 33% of reported bacterial foodborne cases in Norway (1988-1993), over 4-5% in France, to only 2% in the Netherlands (1993-1998). Between 1993 and 1998 there were 278 *B. cereus* outbreaks in Europe, causes 2918 cases (EFSA, 2005a). Presumably the incidence does not differ much between European

countries, and the difference is mainly due to underreporting. The number of foodborne *B. cereus* cases in the USA in 2006 was estimated at 63,400 (90% CrI: 15,770-148,827) (Scallan *et al.*, 2011). This estimate was based on 85 laboratory confirmed cases of foodborne *B. cereus* and a considerable multiplier for underreporting (25.5) and under-diagnosis (29.3). Rajkovic (2006) provided a very comprehensive overview of worldwide *B. cereus* outbreaks (since 1975). The list of implicated products is diverse and long, but the cause is typically mass-catering or home preparation. Just last year, an outbreak of emetic *B. cereus* in a Belgian childcare facility affected 20 children (De Standaard, 2013). In contrast to the large number of mild foodborne illnesses, there is a small number of deaths attributed to *Bacillus cereus* (Takabe & M., 1976; Mahler *et al.*, 1997), of which two deadly cases in Belgium (Dierick *et al.*, 2005; Naranjo *et al.*, 2011). However, the attribution of the most recent case to *B. cereus* remains disputed (Sanaei-Zadeh, 2012; Bottone, 2012). No *B. cereus* cases attributed to industrially prepared REPFEDs have been reported. However, this does not necessarily mean that there are none.

1.2.2 *Clostridium botulinum*

C. botulinum is a Gram positive, rod-shaped, strictly anaerobic, spore forming bacteria, belonging to the same genus as *C. difficile*, *C. perfringens*, *C. sordelii* and *C. tetani*. *C. botulinum* is ubiquitous in the environment and is found in soil, marine sediments, aquatic environments and the gastrointestinal tract of several animals (EFSA, 2005b; Peck, 1997). Although they are usually present in low numbers, the fact that they are so wide-spread means that raw products cannot be guaranteed to be free of *C. botulinum* spores.

C. botulinum produces one of the most potent (if not the most potent) known natural toxins: botulinum toxin. The lethal dose for humans has been estimated at 1 ng per kg body weight (Gill, 1982), the consumption of just 0.1g of food, in which a neurotoxin producing *clostridium* strain was allowed to grow, is sufficient to cause botulism. Although the toxicity of the complete toxin is many orders of magnitude less when consumed orally, the progenitor toxins may actually be more toxic when orally ingested. Possibly because the excess material reduces inactivation in the gut (Gill, 1982). The botulinum toxin causes muscle paralysis and if no action is taken, the afflicted person usually dies from asphyxiation due to paralysis of the respiratory muscle (Peck, 1997). The toxin is relatively heat sensitive and will be inactivated by heating 10 minutes at 80°C (EFSA, 2005b).

Six spore forming anaerobic bacteria, each with distinct physiology and phylogenesis, have the ability to produce botulinum toxin: four groups of *C. botulinum*, *C. baratii* and *C. butyricum* (Peck & Stringer, 2005). Of these six organisms, *C. botulinum* types I and II are responsible

for most of the foodborne botulism cases, their most important characteristics are given in table 1.2. Proteolytic *C. botulinum* (Group I) strains are mesophilic with a minimum temperature for growth and toxin production of 10°C, and a high heat resistance. Because of their high heat resistance, and mesophilic nature, they are mainly a hazard in low acid canned foods. In these products they are controlled using the 'botulinum cook' (F_0=3 or 121.1°C for 3 min) (Peck & Stringer, 2005; Gibbs, 2002). Non-proteolytic (type II) *C. botulinum* strains are more heat sensitive, but are able to grow at refrigeration temperatures. It is precisely this cold-growing ability that makes them a hazard in REPFEDs. Proteolytic *C. botulinum* strains are far less likely to cause problems in REPFEDs, simply because these products are supposed to be stored at refrigeration temperatures. Because the psychrotrophic strains are less heat resistant, the advised pasteurisation treatment is 90°C for 10 minutes (P_{90}=10).

Like *B. cereus*, *C. botulinum* is ubiquitous in the environment and can be present on nearly all foods, but the prevalence on REPFEDs is presumably lower than for *B. cereus*. Del Torre *et al.* (2004) sampled gnocchi, a typical potato based Italian product, for both proteolytic and non-proteolytic *C. botulinum* and found no positive samples. Carlin *et al.* (2004) sampled 372 different REPFEDs in France and reported that 6.6% of the samples tested positive for *C. botulinum*. However, *C. botulinum* concentrations were very low, between 1-3 *C. botulinum*/kg. Broconnier *et al.* (2001) tested 37 samples of raw materials used for REPFEDs production (vegetables, spices, ingredients and texture agents) and 88 vegetable puree samples. None of the 125 samples was positive for *C. botulinum*.

Since the symptoms of botulism are much more serious than for *B. cereus*, the underreporting is probably much smaller and statistics will more accurately reflect the real incidence. Most botulism cases arise from home-canned foods or improperly salted or cooked fish. The number of outbreak in Europe varies between 1 to 5 per year (up to 20 in Germany) (Gibbs, 2002). No outbreaks have been reported in REPFEDs, but some have been reported in REPFED-like products (pasteurised dairy desserts and canned meat). Aureli *et al.* (2000) reported on an outbreak from mascarpone in Italy in 1996, but it is not clear if the cause is recontamination or insufficient pasteurisation. More recently, Juliao *et al.* (2013) reported on a *C. botulinum* outbreak from canned hot dog chilli sauce in the USA (in 2007). It was reported as the first *C. botulinum* case involving a commercial cannery in over 30 years. They were unable to determine the exact cause of the outbreak.

Table 1.2: Characteristics of group I and II *C. botulinum* (Gibbs, 2002; Peck & Stringer, 2005; ICMSF, 1996)

Characteristic	Proteolytic Group I	Non-proteolytic Group II
Neurotoxin type	A,B,F	B,E,F
Minimum growth temperature	10 - 12°C	3 - 3.3°C
Optimal growth temperature	35 - 40°C	18 - 25°C
Minimum pH for growth	4.6 - 4.7	5.0
Minimum a_w (max % NaCl[b]) for growth	0.94 (10%)	0.97 (5%)
$D_{100°C}$ of the spores	>15 - 25 min	< 0.1 min
$D_{121°C}$ of the spores	0.1 - 0.2 min	< 0.001 min
Stability of neurotoxins	$D_{74°C}$ < 3 min, higher at low pH	
Food with potential problems	Low acid canned foods	In pack pasteurised REPFEDs

[b] % NaCl on water phase

1.2.3 *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive (G^+) pathogenic microorganism, which is able to cause various infections (called listeriosis) ranging from mild symptoms, like local skin lesions that are self-resolving or vomiting and diarrhoea, to more series symptoms like meningitis. *L. monocytogenes* is an opportunistic pathogen that affects pregnant women, unborn or newborn children, elderly people and people with a compromised immune system (WHO/FAO, 2004). It is especially dangerous to unborn or newborn babies. Although the mothers' symptoms are usually flu like or even absent the child runs the risk of meningitis and even spontaneous abortion or stillbirth (Bell & Kyriakides, 2002). Foodborne listeriosis is rare, but fatality rates are high (20-30%) in comparison to other foodborne pathogens. Besides food, *L. monocytogenes* has two other infectious routes: contact with animals and cross-infection between new-born babies (Bell & Kyriakides, 2002).

L. monocytogenes is a vegetative (i.e. non spore forming) psychrotrophic microorganism, able to grow between -0.4 and 45°C, with an optimal temperature of 37°C and it is not inactivated during freezing. Its pH (4.4-9.4) and a_w-range (≤0.92) allow growth in most REPFEDs. *L. monocytogenes* grows optimally under micro-aerophilic conditions but grows equally well under

anaerobic or aerobic conditions. Even 30% CO_2 is insufficient to inhibit growth (ICMSF, 1996; Bell & Kyriakides, 2002).

L. monocytogenes is generally considered to be the most heat-resistant foodborne vegetative microorganism (Hansen & Knochel, 2001; Farber & Peterkin, 1991). Inactivation starts at around 55°C ($D_{55°C}$: 8-40 min) but becomes very rapid at temperatures above 70°C ($D_{70°C}$:0.05-0.27 min) (Gaze, 2006). For calculating pasteurisation-values the following values are usually used: $D_{70°C}$: 0.3 min and z-value =7.5°C. The 'classic' heat treatment to inactivate *L. monocytogenes* is 70°C for 2 minutes and guarantees a 6 log (or 6D) reduction in *L. monocytogenes* (ICMSF, 2002).

L. monocytogenes is present in almost any environment and sporadically occurs in most raw materials (Bell & Kyriakides, 2002). It can be considered a pathogen of concern for REPFEDs for two reasons: (i) inadequate thermal processing and (ii) post-process recontamination. The latter can be the case for products that are (re)packaged after pasteurisation and therefore a high care packaging facility is necessary for the safe production of these foods.

Since *L. monocytogenes* is common in the environment, it is inevitably present on raw materials from time to time (Bell & Kyriakides, 2002). Presence of *L. monocytogenes* has been reported in raw chicken, beef, raw milk, shellfish, vegetable salads, raw fish, smoked salmon, ice cream, fermented sausages, aspic, cured beef, loin, luncheon meats, RTE sandwiches and many others (Bell & Kyriakides, 2002; WHO/FAO, 2004; Lianou & Sofos, 2007). The incidence of listeriosis is about 0.3 per 100,000 inhabitants, both in the EU and in the USA. The mortality rate is between 12 an 17% (Lianou & Sofos (2007), EFSA (2011); EFSA (2012); EFSA (2013)). Although *L. monocytogenes* is commonly linked to RTE-foods (e.g. cooked sliced ham), these should not be confused with REPFEDs.

1.3 Effects of thermal processing

1.3.1 A brief history of thermal processing

Thermal processing of food is thought to have begun approximately 0.5 million years ago, although some claims of earlier controlled fires have been made (James, 1989). According to certain researches thermal processing was a fundamental trait, which allowed humans to support their larger brains. Cooked food is easier to digest and this results in a larger net calorie intake (Wrangham & Conklin-Brittain, 2003). Although the reason for this first use of thermal processing remains the centre of debate (Carmody *et al.*, 2012; Wollstonecroft *et al.*, 2012).

However, it is important to note that the primary reason for heating was very likely digestibility and not preservation, until the 18th century salting was used to preserve foods (Albarracin *et al.*, 2011).

Many centuries later, the French emperor Napoleon said: "Une armée marche a son estomac", but even before these famous words were uttered, the french army offered a price of 12,000 francs for the invention of a new food preservation method. As a point of reference: the annual wage was between 350 and 700 francs for a labourer and approximately 1500 francs for a professor in chemistry. After 15 years of research, Nicolas Appert submitted his invention and received the price for what is generally considered the beginning of thermal processing (Appert, 1810). Although the first to patent a thermal preservation method were Procter and White in 1691 (Cowell, 1997), Appert was the first to start with the practical application (Gould, 2006). In the same year, the Frenchmen Pierre Durand was granted the first patent for preserving food in tin cans.

While these inventions marked the beginning of thermal processing, it took another 45 years to discover how the thermal processing worked. The assumption at the time was that the foods preserved longer due to the absence of oxygen. Only when Louis Pasteur disproved the theory of spontaneous generation in the 1850s, it was discovered that microorganisms are responsible for food spoilage. At the same time, Pasteur also patented the first pasteurisation process for the conservation of wine. It took another fifty years (until 1897) to discover that the real cause for spoilage of canned foods were (and are) bacterial spores (Prescott & Underwood, 1897).

Finally in the beginning of the twentieth century Bigelow (1921) and Esty & Meyer (1922) were the first to model the logarithmic destruction of *Bacillus botulinus* (at the time the name for *C. botulinum*) by heat treatment. These first 'models' did not yet use the now so recognisable semi-log charts with D- and z-values. These concepts were introduced later by Katzin *et al.* (1943), Ball (1943) and Stumbo (1949). During the rest of the twentieth century several researchers would build upon these findings to develop the *botulinum*-cook and other safe harbours (see section 1.3.4).

1.3.2 Bacterial spores during and after thermal treatment

1.3.2.1 Spores and the mechanism of thermal inactivation

Bacterial spores are highly specialised cell types, which are designed and able to survive extreme conditions of pH, a_w, temperature and even the vacuum of space. Spore formation (sporulation) is initiated by the depletion of nutrients and begins with the asymmetric division of the mother

cell. The smaller half will develop into an endospore, the larger mother cell will support the spore formation, and once this process is complete the mother cell lyses and the spore is released. The newly formed spore consist of a highly condensed core (with only 30-50% water and high concentrations of dipicolinic acid (DPA)), surrounded by several layers: (i) the inner membrane, (ii) The cortex and germ cell wall, (iii) the outer membrane, (iv) the coat and (v) the exosporium (De Vries, Y.P., 2006). Once conditions become favourable once again, the spore will germinate and form a vegetative cell (De Vries, Y.P., 2006; Markland *et al.*, 2013). This process of germination is usually triggered by the presence of nutrient germinants (e.g. sugars, amino acids) or non-nutrient germinates (dodecylamine, heat treatment, etc.) (Setlow, 2003).

However, not all spores will germinate when conditions become favourable, these are called super-dormant spores (Ghosh & Setlow, 2009a). These super-dormant spores require higher concentrations of germinants, multiple germinants or heat activation (Ghosh & Setlow, 2009b). In addition, Ghosh *et al.* (2009) reported that the wet-heat resistance of super-dormant-spores is higher and that they require higher temperature for heat activation. These super-dormant spores are likely an additional survival mechanism. Spores that germinate more slowly, are more likely to survive if conditions turn unfavourable once again. Their delay in germination increases the survival of the entire population (Markland *et al.*, 2013). The presence of super-dormant spores is the reason that tyndallisation is no longer used as preservation method. During tyndallisation, foods are heated and cooled several times over a period of several days. The idea is that spores will be activated by the first (few) heat treatments, germinate, and that the vegetative cells are then inactivated by the next few heat treatments. The process is rarely used nowadays because of the presence of super-dormant spores (Markland *et al.*, 2013; Gould, 2006). However, attempts are still made to use this combination of heat activation and subsequent heat inactivation (Lovdal *et al.*, 2011).

Until fairly recent, the mechanism by which wet-heat inactivated bacterial spores was unknown (Gould, 2006) It was known that inactivation of spores by dry heat, UV or γ-radiation caused DNA damage. However, wet heat does not inactivate spores via DNA damage, because the DNA is protected by α/β-type small, acid-soluble spore proteins (SASP) (Nicholson *et al.*, 2000). Heat inactivation is often accompanied by inactivation of core enzymes and the disintegration of the spore's inner membrane (Setlow, 2006; Warth, 1980). Coleman *et al.* (2007) reported that wet-heat inactivation of *Bacillus subtilis* damaged one or more key proteins in the spore, which causes inactivation. The protein inactivation is most likely caused by heat denaturation due to the increased water content of the core. This increase in water content is possible due to the release of DPA from the core, although in some cases spores could be dead and still have retained their

DPA. In a more recent study, Coleman *et al.* (2010) confirmed that the same mechanism is valid for *B. cereus*.

1.3.2.2 Parameters influencing thermal inactivation

The thermal inactivation of bacterial spores is affected by a large number of environmental parameters: a_w and pH (Samapundo *et al.*, 2011c; Coroller *et al.*, 2001; Couvert *et al.*, 1999), NaCl concentration (Periago *et al.*, 1998), recovery medium (Gonzalez *et al.*, 1997), heating medium (Juneja *et al.*, 1995), sporulation temperature (Gonzalez *et al.*, 1999; Leguerinel *et al.*, 2007; Baril *et al.*, 2012; Planchon *et al.*, 2011), lysozyme (Scott & Bernard, 1985), spore maturation (Sanchez-Salas *et al.*, 2011), heat activation (Fernandez *et al.*, 2001a), fatty acids in the heating and recovery medium (Lekogo *et al.*, 2010), etc. The effect of these variables on the heat resistance has been extensively documented and modelled (Section 1.3.6). However, van Asselt & Zwietering (2006) performed a meta-analysis on a large number of D- and z-values for different pathogenic microorganisms from literature (n=4066, of which 484 for *B. cereus*, 375 for *C. botulinum* and 967 for *L. monocytogenes*) and concluded that the overall variability between strains is considerably larger than the reported effect of most factors.

1.3.3 Quantification of thermal inactivation

1.3.3.1 Classic log-linear (D-/z-) approach

The traditional approach to pasteurisation or sterilisation uses two parameters to quantify the time-temperature combination required for inactivation. The first parameter is the D-value or decimal reduction time. This time is defined as the time in minutes needed to destroy 90% of microorganisms present in the product. The D_T-value (D-value at temperature T (°C)) is usually determined by survivor studies, in which the log of the number of surviving microorganisms is plotted versus the heat treatment time at constant temperature (Figure 1.4a). The primary model is given in equation 1.1, with N_0 and $N(t)$ the bacterial count respectively at the beginning and at time t(Holdsworth, 2004; Stumbo, 1948). Typical D- and z-values for relevant microorganisms are given in table 1.3.

$$log(N(t)) = log(N_0) - \frac{t}{D_T} \tag{1.1}$$

Note that this approach by Stumbo (1948) (Eq. 1.1), describes the same behaviour as the first order model for bacterial inactivation (Eq. 1.2) (Bean *et al.*, 2012).

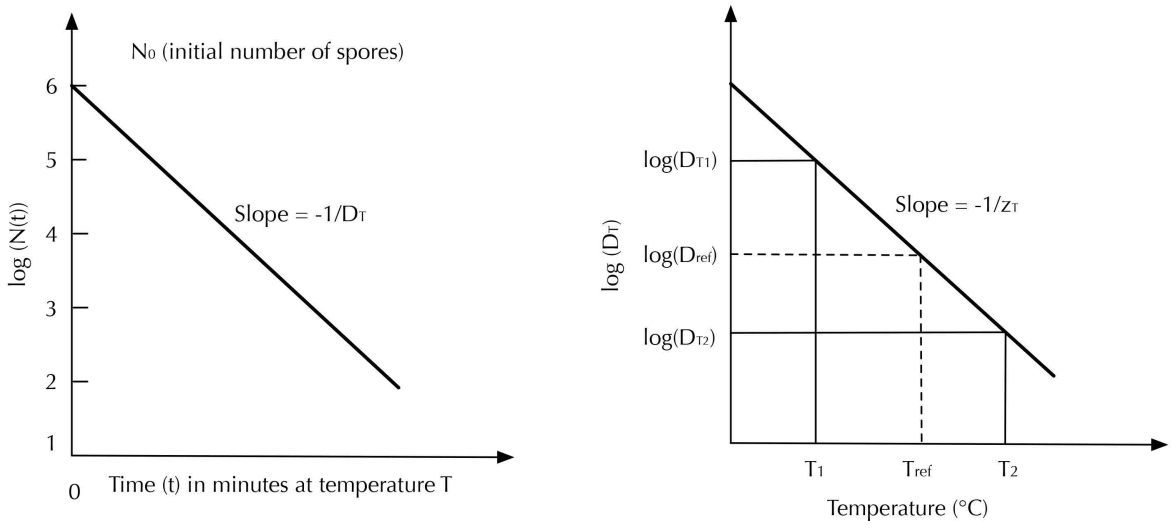
$$\frac{dN}{dt} = -kN \tag{1.2}$$

The second parameter to determine the time-temperature combination needed for inactivation is the z-value. This value is determined by plotting the log of the D_T-value in function of temperature (Figure 1.4b). The secondary model is given in equation 1.3. (Bean *et al.*, 2012; Bigelow, 1921). With D_{ref} the decimal reduction time at a given reference temperature T_{ref} (°C) and z_{T} the temperature increase (or decrease) needed to reduce (or augment) the D-value with a factor 10.

$$log(D_T) = log(D_{ref}) - \frac{(T - T_{ref})}{z_T} \tag{1.3}$$

The D-/z- approach can also be expanded for other parameters than temperature. Gaillard *et al.* (1998) describe an expanded Bigelow-model for temperature, pH and a_w (Eq 1.4). In this equation pH_{ref} is the pH of maximum heat resistance, z_T, z_{pH} and z_{a_w} are respectively the change in temperature, pH and a_w that lead to a ten-fold change in D-value. D_{ref} is the decimal reduction time at T_{ref}, pH_{ref} and a_w 1.

$$D = D_{ref} \cdot 10^{-\left(\frac{T-T_{ref}}{z_T}\right)} \cdot 10^{-\left(\frac{pH-pH_{ref}}{z_{pH}}\right)^2} \cdot 10^{-\left(\frac{a_w-1}{z_{a_w}}\right)} \tag{1.4}$$



(a) D_T-value **(b)** z_T-value

Figure 1.4: Illustration of the determination of the D_T- and z_T-value. (a) Logarithm of surviving microorganisms as a function of heating time, (b) log of D_T values as a function of changing heating temperature (adapted from Holdsworth (2004)).

Table 1.3: Some examples of D- and z-values for a selection of relevant microorganisms (adapted from Bean *et al.* (2012))

Microorganism	T_{ref} (°C)	D_{ref} range (min)	z_T (°C) mean (range)	Ref.
Sporeformer	121.1	0.02 - 5	10 (7 - 12)	(a)
Vegetative cells	70	0.27 - 11	7	(b)
Proteolytic *C. botulinum*	120	0.058 - 0.48	10.2	(c)
Non proteolytic *C. botulinum*	120	0.001 - 0.891	18.6	(c)
B. cereus	120	0.003 - 0.550	12.8	(c)
L. monocytogenes	70	0.014-0.550	7	(c)
L. monocytogenes	70	0.05 0.27	(4.9 - 10.8)	(b)

[a] Holdsworth (2004); [b] Gaze (2006); [c] van Asselt & Zwietering (2006)

1.3.3.2 Alternative approaches

The D-/z- approach is fairly rudimentary, but it is widely used in the food industry as a generally accepted and practical system. However, the graphs presented here (Figure 1.4) are best-case scenarios, a number of bacterial inactivation curves will deviate significantly from these semi-log curves. Two commonly occurring deviations are 'shoulders' and 'tails' (Cerf, 1977; Holsinger *et al.*, 1992; Juneja & Eblen, 2000; Cava-Roda *et al.*, 2012). In the case of a shoulder, the initial inactivation is slower, resulting in a less steep inactivation curve in the beginning (Figure 1.5). In the case of tailing, the initial inactivation follows the log-linear approach but flattens out at a certain point. Causes for tailing may be the method of thermal treatment (e.g. insufficient mixing), a heterogeneous population, heat adaptation (van Asselt & Zwietering, 2006) or the presence of both vegetative cells and spores. When the D-value is determined for mild temperatures (e.g. 60°C) the vegetative cells will be inactivated, but the spores will survive, resulting in tailing (den Besten *et al.*, 2006).

When the inactivation curve has a shoulder or tail, the loglineair (D-/z-) approach does not give a good fit, alternative models may be used such as a log logistic or Weibull model (van Asselt & Zwietering, 2006). Van Boekel (2002) discussed the application of a Weibull model for inactivation curves that have tails or shoulders. He excluded spores, because to the complications posed by the presence of dormant spores that are activated by the heat treatment. Geeraerd *et al.* (2005) even developed a Microsoft® excel add-in for end-users in the food industry. The add-inn can fit nine model types to user-specific data.

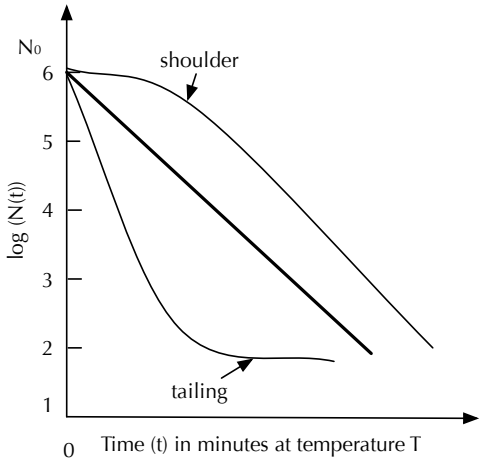


Figure 1.5: Two common types of microbial inactivation curves, which deviate from the 'standard' D-/z-approach

1.3.3.3 P and F-values

The D-value discussed in the previous section are determined at a specific temperature; hence the D_T notation. In order to calculate the heating time required at a certain temperature (T°C) to achieve a certain number of log reduction, it is sufficient to multiply the D_T-value with the number of required reductions. The z-value enables the calculation of the D-value at different temperatures. To determine the heating time needed to achieve a certain inactivation at a different temperature, the lethal rate can be used. The lethal rate is the time needed to achieve an equivalent heat treatment, compared to T_{ref}, at a different temperature (Equation 1.5, Gaze (2006)). With T_{ref} the reference temperature (e.g. 90°C), T the heating temperature (e.g. 85°C) , and t the heating time at T_{ref}. As an example, if z is 9°C, then 1 minute at 85°C corresponds to 0.28 min at 90°C. Note that the unit of L depends on the unit of t (Equation 1.6).

$$L = t \cdot 10^{\frac{(T - T_{ref})}{z}} \tag{1.5}$$

$$L = 1\ [min] \cdot 10^{\frac{(85-90)}{9}} = 0.28\ [min] \tag{1.6}$$

However, the D-/z- approach can only be used for isothermal heating processes, something that is unlikely given the given batch weights and volumes common in industrial food production. If the heating process is non-isothermal the inactivation can be approximated by integrating the legal rate over the thermal treatment. A calculation that can be approximated by summating the lethal

value between measuring intervals (Δt), with T_i the temperature during the measuring interval.

$$L = \int_0^t 10^{\frac{(T-T_{ref})}{z}} dt \tag{1.7}$$

$$L = \Delta t \sum_0^t 10^{\frac{(T_i-T_{ref})}{z}} \tag{1.8}$$

However, this method of extrapolation is preferably not extended outside the range of tested temperatures. Even more important, it should not be taken beyond the limit of biological logic. If temperature decreases, then at some point temperature no longer has an inactivating but a growth stimulating effect. An example: a *L. monocytogenes* strain with $D_{70°C}$=0.33 and z=7.5°C, requires 0.33 minutes at 70°C to achieve a 1 log reduction. Following the definition of z-value, 3.3 minutes at 62.5°C will give the same reduction. And theoretically, 33,000 minutes at 32.5°C will still give the same reduction. However, in reality this temperature will no longer inactivate *L. monocytogenes*, but allow it to grow.

P- and F- values are process values, with F-values used for sterilisation and P-values for pasteurisation (see section 1.3.4 for the difference). A P-value is usually written as: $P^z_{T_\mathrm{ref}}$, with T_ref the reference temperature and z the z-value of the target organism. An example is given in equation 1.9.

$$P^z_{T_{ref}} = P^{7.5}_{70} = 2 \text{ min} \tag{1.9}$$

The $P^{7.5}_{70}$=2 means that the (non-isothermal) heat treatment applied was equivalent (i.e. had the same lethal rate) as an isothermal treatment of 2 minutes at 70°C. For the most common sterilisation or pasteurisation values, the z-value or even the T_ref is no longer included. It is generally understood that F_0 relates to pasteurisation at 121.1°C. In theory this does not apply to pasteurisation processes, which are very flexible (Gaze, 2006), although for some P-values the z-value is rarely mentioned: e.g. P_{90}, P_{70}, P_0 (see section 1.3.4).

1.3.4 Safe harbours, current situation and evolution

The current heat treatments guarantee food safety based on a defined number of decimal reductions (D-values) of a specific target organism. These heat treatments are considered safe harbours (dutch: *pasteurisatie barema's*). This implies that they can be readily used as a processing step, without the need for extensive information about the product's properties or the initial microbial contamination. Safe harbours are usually based on a set of worst case assumptions such as the D-value of the most heat resistant pathogen and the presence of high counts of the pathogen in

the product prior to pasteurisation. In addition the producers must ensure that all parts of the product are processed sufficiently (Fryer & Robbins, 2005). These worst-case assumptions are the reason that safe harbours are significantly fail-safe. This precautionary approach has proven very effective for the industry, but in recent years there is a trend to more minimal processing to achieve higher quality products (more added value). Thermal treatment has many positive effects on food products (e.g. prolonged shelf life, improved digestibility, palatability, etc.), but it also affects the taste, structure, texture and the nutritional value of the product. Therefore, it is an obvious route of improvement (Arnoldi, 2002; Van Boekel *et al.*, 2010)

Commercial sterility is "*the condition achieved by application of heat which renders food free from viable microorganisms, including those of known public health significance, capable of growing in the food at the temperatures at which the food is likely to be held during distribution and storage*" (UK Department of Health, 1994). Given the global difference in 'normal storage temperatures', there is a lot of variability in the processing used to achieve commercial sterility (Anderson *et al.*, 2011). Pasteurisation is a milder form of heat treatment and defined as "any heat treatment which is less than F_0 = 3, but is designed to reduce the numbers of pathogenic and spoilage organisms, and is used in combination with other factors to make foods safer over a designated shelf life" (Gaze, 2006).

Below, three of the most commonly used safe harbour heat treatments are discussed. In addition to these three, many other safe harbours are used in specific industries or for specific products. E.g. 85°C for 4 minutes or 90°C for 10 sec for citrus juices (Robinson *et al.*, 2000) or milk pasteurisation for 30 minutes at 63°C or 15 sec at 72°C (Bean *et al.*, 2012).

1.3.4.1 P_0 =2 / P_{70} =2

The concept of P_0 =2 was originally developed for the treatment of *Coxiella burnetti* in milk. The highest level of *C. burnetti* detected in raw milk was 10,000 infective guinea pig doses, and the goal was to provide a 10 fold safety margin. A heat treatment of 20 seconds at 71°C (160°F) was deemed necessary to achieve this (Enright *et al.*, 1957; Bean *et al.*, 2012). In the 1980s, *L. monocytogenes* became the primary focus of the dairy industry. It was determined by Farber *et al.* (1988) that *L. monocytogenes* could be eliminated (6D) by heating the milk 15 seconds at 75°C. For other products, the $D_{70°C}$-value ranged from 0.14 to 0.27min. Hence a two minute heat treatment at 70°C was advised ($6 \cdot 0.27$=1.62 + safety margin) (Gaze *et al.*, 1989). Since *L. monocytogenes* is generally accepted as the most heat resistant vegetative pathogen (Farber & Peterkin, 1991; Hansen & Knochel, 2001; Mackey *et al.*, 1994), this treatment is now used to ensure inactivation of all vegetative pathogens.

1.3.4.2 P_{90} = 10

This safe harbour is designed to achieve at least a 6D reduction of psychrotrophic non-proteolytic *Clostridium botulinum* strains (Group II) and is therefore also called the "non-proteolytic *C. botulinum* cook' (Membré *et al.*, 2009).These *C. botulinum* strains are the specific target organisms for mitigation of spore-forming pathogens. This safe harbour was established by the Advisory Committee on the Microbiological Safety of Food in 1992 (ACMSF, 1992) and is also used for mitigation of *B. cereus* (Carlin *et al.*, 2000a). The time-temperature combination is based on the $D_{90°C}$-value (1.1 min) of a single *C. botulinum* strain isolated from cod. A simple 6D reduction would thus require 6.6 minutes at 90°C, but the safe harbour includes a 3-min safety margin for variations in strain heat resistance (ACMSF, 1992; Gaze & Brown, 1990). The z-values used for calculation of process equivalence are temperature dependent. The z-value is usually set at 7°C for temperatures below 90°C and at 10°C for temperatures above 90°C (ECFF, 2006).

1.3.4.3 F_0=3

The F_0=3 or 3 minutes at 121.1°C (250 °F) is designed for low-acid (pH>4.5) canned foods and corresponds to a 12 log reduction in proteolytic *C. botulinum*, hence the name 'botulinum-cook'. The value of three minutes is based on the data by Esty & Meyer (1922), and the concept was developed by Bigelow (1921) and Stumbo (1965). The $D_{121.1°C}$ was determined to be 0.21 min, hence the time needed for a 12-log reduction was 2.52 min. With an additional safety margin, this became the F_0=3. This treatment actually corresponds to a 14D reduction in *C. botulinum* spores (3/0.21=14.3) (Bean *et al.*, 2012).

1.3.5 Sublethal injury

Heat treating bacterial spores does not only inactivate a certain amount of spores, but also causes injury to the spores that are not inactivated. These injuries are usually structural or metabolic and are reversible. The injured spores require more or different nutrients before they can grow and need more time to recover, which will manifest as longer lag times (Adams, 2005). Hence, when they are put in suitable conditions, the microorganisms will recover (Lund & Peck, 1994; Adams, 2005). Peck (1997) stated that a probable type of injury to spores, is damage to the germination mechanism. This prevents the surviving spores from returning to their active physiological state. This can also be the reason that lysozyme appears to increase spore heat resistance. Some bacterial spores are permeable to lysozyme and the lysozyme hydrolyses the peptidoglycan cortex of

the spores, enabling germination (Peck & Fernandez, 1995). More recently, Coleman & Setlow (2009) suggested that the injury does not prevent germination, but outgrowth. Since the injured microorganisms are not always detected using classical plate counts methods, it is important to use adequate methods to resuscitate the injured cells (Nyachuba & Donnelly, 2005).

The current safe harbours only consider microbial inactivation (log reduction). Membré *et al.* (2009) presented a concept to incorporate the effect of sublethal injury in the classic safe harbour approach. The 'Degree of Protection' (DoP) combines inactivation and thermal injury (equation 1.10).

$$DoP = \Delta R + ThI \tag{1.10}$$

In this equation ΔR is the log reduction and ThI is the thermal injury. Both are expressed as the decimal logarithm of the reciprocal of a probability (equations 1.11 and 1.12).

$$\Delta R = log\left(\frac{1}{P_r}\right) \tag{1.11}$$

$$ThI = log\left(\frac{1}{P_i}\right) \tag{1.12}$$

With P_r the probability that a spore survives the heat treatment and P_i the probability that the lag time is shorter than the storage time. Using this approach, Membré *et al.* (2009) constructed an exposure assessment for non-proteolytic *C. botulinum*. And used it to determine a set of shelf life and pasteurisation combinations that gave the same probability (i.e. iso-probabilities) for *C. botulinum* growth during shelf life. They reported that a combination of pH 6.3, shelf life 30 days and pasteurisation for 10 min at 88°C, gave the same probability ($1 \cdot 10^{-6}$) of having non-proteolytic *C. botulinum* as pasteurising 10 min at 84°C, for a product with pH 5.7 and a shelf life of 50 days. The incorporation of this sublethal injury by the DoP, will allow producers to determine less intense heat treatments that yield the same level of food safety.

1.3.6 Requirements of a model for spores in REPFEDs

For REPFEDs there is a trend towards minimising the heat treatment. However, it is imperative that the microbiological food safety of the product is guaranteed. One method to assess the possibilities for growth as a function of heat treatment is predictive microbiology.

To describe the situation of a bacterial spore former in REPFEDs, a predictive microbiological models must fulfil three criteria:

1. The model must be designed with spores, because vegetative cells will not survive the pasteurisation treatment (Byrne *et al.*, 2006)

2. The model must incorporate a heat treatment, because this will affect both lag time and growth (Gaillard *et al.*, 2005)

3. Lag time and growth should be measured under cold storage, because this is standard practice in the industry and it will affect the lag time and growth rate (Choma *et al.*, 2000a).

In the case of *B. cereus*, none of the existing models fulfil all three criteria. Some models predict growth at low temperatures, but only for vegetative cells without heat treatment (e.g. Baker & Griffiths (1993); Olmez & Aran (2005)). Other models use spores and apply a heat treatment, but either only predict inactivation (e.g. Collado *et al.* (2003a); Samapundo *et al.* (2011c)) or cannot be used at cold temperatures (e.g. Gaillard *et al.* (2005); Laurent *et al.* (1999)). Membré *et al.* (2009) described a square root model for *C. botulinum* fulfilling all three criteria but did not publish the value of the model parameters. Tables 1.4 and 1.5 present an overview of the available models for growth and inactivation of *B. cereus*, with indication of which of the three criteria is not fulfilled.

Table 1.4: Available predictive models for *B. cereus* lag time or growth rate

Reference	Model output	Parameters [range]	Medium/product	Unfulfilled criteria
Baker & Griffiths (1993)	Time to detection (hours)[a] Vero [b] RPLA [c]	Incubation temperature [6 - 38°C] pH [5.8 - 8.0] a_w (0.965 - 0.995) Starch [0 - 0.625%] Glucose [0 - 1.8%]	BHI[d] broth	1 & 2
Quintavalla & Parolari (1993)	Time to visible growth (days)	Temperature [11.5 - 28.5°C] a_w [0.91 - 0.96] pH [4.2 - 7.0]	Tryptose Casein Soy Broth	1 & 2
Benedict *et al.* (1993)	Growth rate (hr^{-1}) Generation Time (h) Lag time (h)	Temperature [5 - 42°C] pH [4.5 - 7.5] NaCl [0.5 - 5%] $NaNO_2$ [0 - 200 mg/l]	BHI	1 & 2
Bennik *et al.* (1995)	Specific growth rate (h^{-1})	Headspace CO_2 [0 - 50%]	BHI agar	1, 2 & 3
Chorin *et al.* (1997)	Growth rate (CFU/hour) Lag time (hours) Concentration after storage (log CFU/ml)	Incubation temperature [20 - 30°C] pH [5.5 - 7.5] a_w [0.95 - 0.99] Initial inoculum size (CFU/ml) Storage time (hours)	BHI broth	1,2 &3

Continued on next page

Table 1.4 – continued from previous page

Reference	Model output	Parameters [range]	Medium/product	Unfulfilled criteria
Laurent *et al.* (1999)	lag time (hr.)	Heating temperature [90 - 100°C] Heat treatment time [1 - 45min] pH of recovery medium [5 - 7] Recovery temperature [25 - 38°C]	Distilled water TSB[e] acidified with Lactic of acetic acid	3
Choma *et al.* (2000a)	Max. specific growth rate (h^{-1})	Incubation temperature [5 - 40°C]	Courgette broth J broth	1 & 2
Lanciotti *et al.* (2001)	Growth/no-growth boundary	Temperature [17.5 - 40°C] a_w [0.915 - 0.990] pH [4.75 - 7.0] Ethanol [0 - 2 %(v/v)]	BHI broth	1,2 & 3
Olmez & Aran (2005)	Lag time (hours) Growth rate (Ln/hr) Concentration after storage (log CFU/ml)	Incubation temperature [8 - 32°C] pH [5.3 - 7.3] Sodium lactate [0 - 600mM] NaCl [85 - 600mM] Storage time (hours)	BHI broth	1 & 2
Gaillard *et al.* (2005)	lag time (hr.)	Heating temperature [85 - 95°C] Heating time [1-50min] pH of recovery medium [5 - 7]	Biokar Nutritive broth Tryptone salt broth	3

Continued on next page

Table 1.4 – continued from previous page

Reference	Model output	Parameters [range]	Medium/product	Unfulfilled criteria
Heo *et al.* (2009)	Growth rate (hr^{-1})	Storage temperature [10 - 40°C] [5.4 - 6.8]	Cooked rice	1 & 2
Bae *et al.* (2011)	Growth rate (hr^{-1}) Lag time (h)	Temperature [15 - 35°C] Seasoning (with / without)	Blanched Spinach	1,2 & 3

[a] time taken for optical density to reach a level of $0.2 = 3 \cdot 10^6$ cells per ml

[b] Vero = diarrheoal toxin production measured using Vero (African Green Monkey kidney) cells

[c] RPLA = diarrhoael toxin production measured using the BCET Reverse Passive Latex Agglutination kit from Oxoid

[d] BHI, Brain Heart Infusion, [e] TSB, Tryptone soy broth

Table 1.5: Available predictive models for *B. cereus* inactivation or survival

Reference	Model output	Parameters [range]	Medium/product
Gaillard *et al.* (1998)	Heat resistance (D-value)	pH [4.5 - 6.5] a_w [0.80 - 1.0] Heating temperature [85 - 105°C]	citrate-phosphate buffer
Couvert *et al.* (1999)	Heat resistance ($D_{95°C}$-value)	pH of heating medium [5 - 7] pH of recovery medium [5 - 7]	citrate-phosphate buffer Nutritive agar (NA)

Continued on next page

Table 1.5 – continued from previous page

Reference	Model output	Parameters [range]	Medium/product
Fernandez *et al.* (1999)	D-value (min) Surviving spores (log CFU) Log reductions	Heating temperature [85 - 105°C] Initial number of spores (CFU) Heating time (minutes)	Double distilled water
Fernandez *et al.* (2001a)	D- and z-value Germination	(non)-isothermal (In)activation Activation heating rate [0.5 - 2°C/min] Inactivation heating rate [0.5 - 2°C/min]	Double distilled water recovery on NA
Fernandez *et al.* (2001b)	surviving spores (log CFU) Log reductions	Initial heating temperature [80 - 100°C] Initial number of spores (CFU)	Double distilled water
Fernandez *et al.* (2002)	Surviving spores (log CFU) log reductions	Heating temperature [80 - 95°C] pH [4.2 - 6.2] Initial number of spores (CFU) Heating time (min)	Carrot broth acidified with citric acid
Collado *et al.* (2003a)	Heat resistance (D-value)	Heating temperature [90 - 105°C] pH [4.7 - 6.2]	Carrot Juice
Leguerinel *et al.* (2007)	Spore heat resistance (D-value)	Heating temperature [92 - 115°C] Sporulation temperature [20 - 45°C]	tryptone salt broth

Continued on next page

Table 1.5 – continued from previous page

Reference	Model output	Parameters [range]	Medium/product
Lekogo *et al.* (2010)	D-value	Palmitic acid	Nutrient Broth
		Palmitoleic acid [0 -2mM]	tryptone salt broth
		Stearic acid [0 -2mM]	
		Oleic acid [0 -2mM]	
		Heating temperature [90 - 100°C]	
Gao & Ju (2010)	Surviving spores ($\log \frac{N_0}{N_t}$)	pH [4 - 10]	0.85% NaCl solution
		Soybean protein [1.67 - 5.0 %(w/w)]	
		Soybean oil [3.33 - 10.0 %(w/w)]	
		Sucrose [0 - 10.0%(w/w)]	
Samapundo *et al.* (2011c)	Heat resistance ($D_{85°C}$-value)	pH of heating medium [5.11 - 7.10]	TSB
		a_w of heating medium [0.931 - 0.981]	Béchamel sauce

1.4 Food safety aspects of consumer behaviour

The microbial safety on REPFEDs is not exclusively governed by the producers or retailers. A large section of the product's shelf life is passed in a consumer refrigerator. A number of consumer practices contribute to the microbial food safety of REPFEDs. Some consumer behaviour can even negate the efforts made to produce safe foods during the production process (Kennedy *et al.*, 2005).

Nissen *et al.* (2002) stated that the health risk associated with sous vide-processed ready meals is small, as long as the storage temperature is low. It is generally accepted that the temperature during production and internal storage at the company is well controlled. However, the temperatures during transport, in retail display cabinets and especially in consumer refrigerators are less controlled (EFSA, 2007; Afchain *et al.*, 2005). The maximum temperature at retail level is usually regulated nationally or supranationally (e.g. on EU level), and the maximum temperatures vary between countries and regions. For example the maximum legal retail temperature in Finland, Sweden and the UK is 8°C, in Belgium 7°C, in Denmark 5°C and in France ≤4°C. Despite being regulated, not all retail display cabinets or transports respect the regulation (Peck *et al.*, 2006). When it comes to food safety, consumers are increasingly considered to be the weakest link, but it would be impractical to legislate and control the domestic storage of food products (Terpstra *et al.*, 2005).

Information about the production and distribution chain is available, but less is known about the consumer practices (Terpstra *et al.*, 2005). A reason for this data-abundance is the strict regulation, the implementation of Good Manufacturing and hygiene Practices (GMP/GHP), prerequisite programs (PRPs) and Hazard Analysis Critical Control Point (HACCP) plans. In addition there is a legal obligation for each producer, to control, ensure and verify that the food products produced under his control satisfy the legal requirements (Anonymous, 2002). The potential for consumer behaviour to cause foodborne diseases, it not to be underestimated. Redmond & Griffith (2003) reported that, depending on the country, between 10% and 87% of the reported foodborne diseases were associated with food prepared at home.

With respect to REPFEDs, several parameters of consumer behaviour affect the exposure to psychrotrophic bacteria. First, the time and temperature of transport from the retail store to the consumer's house. Second, the time and temperature of storage in a consumer's refrigerator. Third, the consumer's respect for the 'use by' date. Fourth, reheating practices and correct use of the microwave. Fifth, the practice of keeping and reheating leftovers. Each of these five is discussed below.

1.4.1 Transport from retail store to the consumer's home

Some data are available for the time of transport by the consumer. Evans *et al.* (1991) (in Nauta *et al.* (2003)) reported data for the UK (mean 42.8 min, st. dev. 18.7min). By comparison, data for the Netherlands shows much shorter times (mean 7.9, st. dev. 5.9) (Voedingscentrum (1999) in Nauta (2001)). More recently, Goldwin & Coppings (2005) reported similar data for the USA, with ±80% of consumer taking less than 20 minutes to return home from the grocery store. They also reported that only 7% of consumers used icepacks or coolers when transporting food products, but this percentage increased as the distance (and drive time) was longer. Jevsnik *et al.* (2008) found similar numbers, for Slovenian consumers: only 15.3% (n=1030) used an isolated bag or cooling box. Kennedy *et al.* (2005) reported that 58% of the Irish households participating in their survey (n=1,020) stored raw meat in the refrigerator less than 30min after shopping, but also that in 7% of the participating households this took more than 90 minutes. The US FDA reported that the mean transport time in the US was 1h05m (US FDA, 1999).

For the temperature during transport from the store to the consumer's home, data are even scarcer. In an exposure assessment of *B. cereus* in a broccoli based REPFEDs, Nauta (2001) used an expert opinion: minimum 4°C, most likely 10°C and maximum 25°C. Kim *et al.* (2013) monitored the temperature of various food products stored in a car trunk, exposed to (summer) sunlight for up to three hours. They reported trunk temperatures between 32.3 and 41.5°C and product temperatures between 33.5°C to 38.4°C (depending on the product). Temperature changed as a function of cloud coverage and solar radiation, yielding product temperature in excess of 40°C. However, after 30 minutes of storage in the trunk, temperatures did not exceed 20°C. The US FDA also measured the mean temperature increase of the food product, based on the time the product was out of refrigeration. After 15 min to 1 hour, the product temperature had increased with 4.5°C (8.1°F). After two hours, product temperature was up by 6.7°C (12.2°F). The mean product temperature before putting it in the consumer's refrigerator was 10.2°C (US FDA, 1999).

1.4.2 Storage in a consumer refrigerator

Contrary to the information about transport, there is a considerable amount of information available about the temperature in the consumer refrigerator, but very little actual data about the time a product spends in a consumer refrigerator. For the refrigerator temperature data is available for the United Kingdom (Evans *et al.*, 1991; Johnson *et al.*, 1998), France (Derens *et al.*, 2004; AFF, 2000), Greece (Sergeledis *et al.*, 1997), the Netherlands (Notermans *et al.*, 1997), Ireland (Kennedy *et al.*, 2005), Belgium (Debacker *et al.*, 2007) and the USA (Pouillot *et al.*, 2010). Generally, the mean refrigerator temperature in Europe is around 6-7°C, and the 75th percentile

(the value typically used in challenge tests) is around 8-9°C. A survey of 1,020 Irish households by Kennedy *et al.* (2005), indicated that no less than 67% of participants were unsure about the correct operation temperature of the refrigerator and that 77% did not have a thermometer in the refrigerator.

A number of studies have been done to determine the time a product spends in a consumer fridge. Pouillot *et al.* (2010) determined the time RTE-products (e.g. deli-meats, sausages, smoked seafood) were stored in a consumer refrigerator. For the time to first consumption (i.e. first opening of the packages), they reported that 50% was consumed between 1 to 5 days and that 90% was consumed within 5 to 15 days (depending on the product). For the time to last consumption (i.e. when the package is empty) they reported much longer times, with 50% being consumed after 7 to 14 days and 90% after 12 to 46 days. Garrido *et al.* (2010) made similar calculations for the storage of smoked fish and packaged meat products, but did not report percentiles. For REPFEDs no survey data is available. In their exposure assessment for *B. cereus* in a vegetable puree REPFEDs, Nauta *et al.* (2003) used an exponential distribution with the 'use by' date as the 95^{th} percentile, based on expert opinion.

Several studies have suggested there might be a correlation between storage temperature and storage time in the consumer fridge (Domenech *et al.*, 2012; Garrido *et al.*, 2010; US FDA, 2003). The assumption behind this correlation is that storage at higher temperatures will cause the product to spoil faster and thus it is less likely to be consumed (US FDA, 2003). Domenech *et al.* (2012) make this assumption based on expert opinion, Garrido *et al.* (2010) make it based on a survey with only 33 consumers. In contrast, Pouillot *et al.* (2010) found no evidence of such a correlation using a much larger dataset. They pointed out that further research into this correlation is necessary, because it will have a considerable impact on quantitative risk assessments.

1.4.3 Respect for the 'use by' date

It is unlikely that all products are consumed before the 'use by' date that is specified on the packaging, however there is little data available about this type of consumer behaviour. Redmond & Griffith (2003) reviewed different food safety studies, but none of these inquired about the 'use by' date. Two studies discuss the consumer's respect for the 'use by' date; Unklesbay *et al.* (1998) surveyed the attitudes and practices of 824 US college students with respect to food safety and noted that most student respected the 'use by' date. Interestingly, he also noted that agricultural and engineering students had less respect for the 'use by' date. More recently, Ergönül (2013) did a survey of 600 Turkish consumers. During the survey, 83% of the participants

indicated they never consumed the food products after the 'use by' date. A recent survey of Belgian consumer by Van Boxstael *et al.* (Under Review) showed that 80% of the participants were familiar with *'best before' date*- and *'use by' date* -labelling and that 70% actually knew the difference between both labels. However, only half of the participants took the difference between these two type of dates in account when assessing food edibility. In addition, participants were flexible in interpreting the 'use by' date, 34.7% indicated they would sometimes eat expired refrigerated RTE products at home.

In his exposure assessment for *B. cereus*, Nauta (2001) assumed that 5% of the product was consumed after the 'use by' date. Domenech *et al.* (2010) assumed, in a case study for pasteurised milk, that all products are consumed before the 'use by' date. The NACMCF (2005) stated that for every well-documented case of *B. cereus*, time or temperature abuse had allowed low levels of *B. cereus* to grow and added that adequate storage temperatures provide sufficient control. They concluded that because of this need for time/temperature abuse, the 'use by' date will have little effect on the exposure. Besides a lack of respect for the 'use by' date, the reason for consuming a product after the 'use by' date might also be more banal. Johnson *et al.* (1998), reported that the majority of elder people understood the 'use by' date or 'sell by' date labels, but that 45% of the elder consumers has difficulties reading them.

1.4.4 Reheating practices

This specific kind of consumer behaviour is more relevant for vegetative pathogens like *L. monocytogenes* than for spore forming pathogens. Although some surveys included the reheating of products, no temperature data are available. Cates *et al.* (2006) reported that 99% of the 1,212 US participants to their survey reheated Frankfurter sausages prior to consuming them. The US FDA (1999) measured the reheating temperatures of several products after consumer-reheating and reported that the mean temperature for a pre-cooked food was 64.2°C (148.2°F), with a standard deviation of 13.7°C (24.7°F). However, temperature ranged from 36 to 96°C, with 34% of the pre-cooked products not reaching a temperature of 60°C during reheating.

1.4.5 Storing and reheating leftovers

The Irish Food Safety Promotions board advises consumers to refrigerate leftovers within 2h after cooking, to use them within 3 days after storage, to only reheat them once and to make sure they are reheated until they are "*piping hot*" (Brennan *et al.*, 2007). However, in reality, this is not always the case; Kennedy *et al.* (2005) reported that 72.2% participants stored meat leftovers (n=1020). Of this group, 79.2% stored the leftovers in the refrigerator, 4% stored them in the

freezer and 16.7% stored them at room temperature (either on the tabletop, in the oven or in a cupboard). When the same group was asked to describe the temperature to which they had most recently reheated leftovers, 58% chose "hot", 14% chose "warm" and 28% chose "cold". For REPFEDs, there is no data available, but it is likely that on some occasions part of a REPFEDs will be kept for later consumption.

1.5 Exposure assessments for *B. cereus* in REPFEDs

Several previous exposure assessments have been done for *B. cereus* in REPFEDs. Most notable is the exposure assessment for *B. cereus* in broccoli puree by Nauta (2001). This exposure assessment served as a case study for a new risk modelling methodology: the Modular Process Risk Model or MPRM (Nauta, 2002). It comprised the entire production process and shelf life of the product, up to the point it is taken out of the fridge by the consumer. In addition, it considered five different *B. cereus* strains and various temperature profiles for consumer storage. Nauta (2001) also identified a number of key data gaps, such as: (i) growth/inactivation models with variability included, (ii) useful model for sporulation, germination and spoilage and (iii) data about consumer behaviour (transport, storage and preparation). The exposure was estimated, depending on the strain, between 0 and 6.5% of the packages contained more than 10^5 CFU/g. However, given the lack of a dose-response distribution, the effect on public health is unsure. The most important conclusions were: decontamination of ingredients may be a good risk mitigation strategy; end-product testing is a bad predictor for consumer food safety risk; prevalence is higher for mesophilic strains, but psychrotrophic strains cause more exposure.

The QMEA for *B. cereus* in courgette puree by Afchain *et al.* (2008) also uses the MPRM methodology. The goal was to determine the exposure to the seven different genetic groups of *B. cereus* identified by Guinebretière *et al.* (2008). They concluded that the proportion of the groups in the total *B. cereus* contamination strongly depends on the processing, storage and possible routes of contamination. They noted that mesophilic groups prevailed, but that they had a much lower growth potential compared to psychrotrophic groups. These psychrotrophic groups had lower prevalence, due to their lower heat resistance, but had higher growth potentials. The virulence properties of the different groups were identified as an additional data gap.

Malakar *et al.* (2004) also performed a QMEA for *B. cereus*. It used the same information as Nauta (2001), but a Bayesian belief network. They reported that the proportion of psychrotrophic and mesophilic strains had the strongest influence on spore prevalence. The assessment did not

include consumer behaviour. Several other exposure assessments are available for *B. cereus* in other products like pasteurised milk, a milk based beverage and RTE Kimbab (rolled cooked rice) (Pina-Perez *et al.*, 2012; Bahk *et al.*, 2007; Notermans *et al.*, 1997; Delignette-Muller & Rosso, 2000).

1.6 Food safety metrics

Food safety at company level is currently controlled by GMP/GHP, PRPs and finally HACCP. Developed in the early 1970s, HACCP was a major improvement in the safe production of food and is product, company and even process specific. It focusses on the hazards likely to affect consumer health and particularly on specific points in the production process, where a loss on control would cause the largest risk for the consumer. To properly function, HACCP requires a solid foundation of GMP, GHP and PRPs. These less specific practices and programs reduce the number of hazards to something that is manageable using HACCP (Van Schothorst, 2004)

However, HACCP is plant specific and makes no link between the effectiveness of the measures taken and the expected level of public health impact (e.g. because HACCP is used, the number of *Salmonella* outbreaks will decrease by 10%). To link food safety requirements and public health impact, a number of concepts have been developed. First, a public health goal must be set by the government. This goal should be based on both scientific and societal factors (e.g. costs of reformulation and surveillance, availability of products, medical costs, etc.). The risk a society (or its government) is willing to accept is the **Appropriate Level Of Protection (ALOP)**. This concept was created by the WTO to permit member states to promote food safety without imposing (illegal) trade barriers. The ALOP is defined as: “The level of protection deemed appropriate by the Member (country) establishing a sanitary or phytosanitary measure to protect human, animal or plant life or health within its territory.”. It is sometimes also referred to as the “acceptable level of risk” (World Trade Organization, 1995; Gorris, 2005). A hypothetical example of an ALOP for *B. cereus* is: “The number of *B. cereus* cases should not exceed 1 per 10,000 inhabitants per year”.

However, making sure that an ALOP is obtained, is not the responsibility of one single food business operator (FBO). It is the responsibility of all the FBOs that produce food products containing that specific pathogen. Hence it is very difficult, if not impossible for a FBO to translate the ALOP to something measurable and useable at company level. To allow this translation the **Food Safety objectives (FSO)** were developed (ICMSF, 2006). An FSO is “The maximum frequency and/or concentration of a (microbial) hazard in a food at the time of consumption that

still provides the ALOP" (ICMSF, 2002). The establishment of an FSO from an ALOP should preferably be done using Microbial Risk Assessment (MRA), either by an expert panel or by a quantitative microbial risk assessment (QMRA). An FSO should be achievable, and if this is not the case, then the production process, the product or the FSO should be modified. If modification of any of these is not possible, the product or process may have to be banned (ICMSF, 2002). It is important to note that while an FSO specifies the target (e.g. no more than 10^5 CFU/g), it does not specify how this should be achieved. This allows the FBO the flexibility to use the method best suited for their situation (ICMSF, 2006). Havelaar *et al.* (2004) suggested a series of improvements to the FSO concept, first that an FSO should be established using a QMRA and not by calculating back from the ALOP. The use of a QMRA also allows the determination of possible control measures. Second, that the FSO should report both maximum prevalence and dose of a microbiological hazard. Because various combinations of these two can yield the same risk. And, that interaction between governmental and industrial risk managers is needed to agree on a risk model.

In some cases the maximum concentration of the hazard in an FSO (e.g. absence in 25g) or the prevalence of the hazard in an FSO (e.g. only 1 in 10^{12} cans may contain viable *C. botulinum* spores) will be very low. This makes it difficult (if not impossible) to detect and control the hazard at this level of the production process. To solve this problem **Performance Objective (PO)** can be set at earlier points in the food chain. A PO is basically the same as an FSO, but set at an earlier time in the production process. A hypothetical example can be: "to assure absence of the hazard in the product after pasteurisation, the concentration before pasteurisation should not exceed 100 CFU/g". It is important to note that both the FSO and the PO must be realistic and achievable and while they are called 'objectives', they are not minimum requirements but maximum tolerable levels (ICMSF, 2006; Gorris, 2005).

While an FSO or a PO may already be more comprehensible and manageable for a FBO, they still have to be translated to **control measures** in the production process. For example: "selection of suppliers to reduce the initial contamination" or "Application of a heat treatment to reduce the number of spores present". The outcomes of these control measures should be specified by **Performance Criteria (PC)** and **Process criteria (PrC)**. The PC specify the desired performance of a control measure: "e.g. achieve a 12D reduction of *C. botulinum* in low-acid canned foods". The PC in turn, is translated to a production level by the PrC. The PrC are the most basic, and probably the most recognisable step. A PrC of the previous 12D PC can be: "heat for 3 minutes at 121.1°C." (ICMSF, 2002). As an example Table 1.6 gives an example of FSO/PO etc. for *C. botulinum* in low-acid canned foods and *L. monocytogenes* in RTE foods. For *B. cereus*, there is

currently no ALOP or FSO available.

If there is no FSO, **default criteria** may be established for specific control measures. These default criteria are fail-safe criteria, developed by expert groups or regulating authorities. They are intended to control the hazard under worst-case conditions and will therefore be less flexible. An example of such a default criterion is the in pack pasteurisation of REPFEDs for 10 minutes at 90°C (ICMSF, 2002; Advisory Commitee on the Microbiological Safety of Food (ACMSF), 1992; Gould, 1999).

Table 1.6: Theoretical example of food safety metrics used for *C. botulinum* in low acid canned foods and *L. monocytogenes* in RTE-Foods, derived from the '12D botulinum cook' and Commission regulation (EC) No 2073/2005 (Anonymous, 2005)

Metric	Example for *C. botulinum*	Example for *L. monocytogenes*
Food Safety Objective (FSO)	No growth of *C. botulinum* in all but one of 10^{12} cans	$< 10^2$ *L. monocytogenes*/g at the moment of consumption
Performance Objective (PO)	No viable *C. botulinum* spores in all but one of 10^{12} cans	Absence in 25g after production, or if growth potential during shelf life is determined (e.g. 1 log), than maximum concentration that will still assure FSO.
Control measure	All cans must be heat treated	Pasteurisation and/or prevent recontamination
Performance criterion (PC)	Heat treatment must cause 12 log reduction	Heat treatment must cause 6 log reduction
Process criteria (PrC)	3 minutes at 121.1°C	2 minutes at 70°C

1.7 Conclusions

This review of scientific literature and visits to the REPFED production sites illustrate the complexity of the microbial food safety of these products. Even if the product are characterised based on the production process or based on the reheating at consumer level, there is a large product diversity. The *B. cereus* strains of concern are (moderately) heat-resistant and psychrotrophic, which they need to be to survive the pasteurisation process and to grow during the product shelf life. The pasteurisation process that is currently used (P_{90} =10min), is based on historical knowledge, a number of worst case assumptions and does not consider the currently used preventive approach or the implementation of PRPs and HACCP. Despite the fact that pasteurisation has been used to assure food safety for centuries, some aspects of the inactivation mechanism are still unknown, other aspects are known, but are not yet included in the determination of the required heating time or temperature (e.g. sublethal injury). In addition to producers, consumers also have a considerable impact on the growth probabilities of pathogenic bacteria in REPFEDs. Especially when it comes to respecting storage times and temperatures. Finally, the current exposure assessment for *B. cereus* in REPFEDs all focus on one product and do not take heat-injury of spores into account. The development of a QMEA for *B. cereus* in REPFEDs is multifaceted and will include basic microbiology (e.g. sampling and analysis), predictive microbiology (e.g. modelling) and consumer behaviour studies (e.g. storage time).

Chapter 2

Current microbial safety & quality of REPFEDs at industrial and consumer level

Redrafted after:

Daelman, J., Jacxsens, L., Devlieghere, F. & Uyttendaele, M. (2013b). Microbial safety and quality of various types of cooked chilled foods. *Food Control*, 30(2):510–517

Daelman, J., Jacxsens, L., Lahou, E., Devlieghere, F. & Uyttendaele, M. (2013c). Assessment of the microbial safety and quality of cooked chilled foods and their production process. *International Journal of Food Microbiology*, 160(3):193–200

Summary

In this chapter the current microbial safety and quality of REPFEDs is evaluated. Historical data on the microbial contamination of REPFED were collected from five companies (n=3168) and samples were taken from the entire production process of the same companies: from raw materials over environmental samples to end products (n=699). In addition, the temperature profile of 38 products was monitored during reheating and a *Listeria monocytogenes* challenge test of paella was performed including reheating. Only a small number of the historic samples exceeded the acceptable levels for *Bacillus cereus* or sulphite reducing *Clostridia* (0.7 / 2%) and no samples exceeded the acceptable levels for *L. monocytogenes*. During production both food contact surfaces (90/226) and gloves (43/92) contained elevated levels of total psychrotrophic aerobic counts (≥3 log CFU/25cm^2). But, only a few (4/223) food contact surfaces and none of the gloves sampled were positive for *L. monocytogenes* (per 25 cm^2). However, none of the end products were positive for *L. monocytogenes* and only 9 end products (6.7%) contained (low numbers of) *B. cereus* (< 2.7 log CFU/g). The pasteurisation values obtained during reheating are very heterogeneous. Only 7 products obtained a P_{70} of 2 minutes throughout the entire product. The challenge test demonstrated that reheating the paella does not guarantee absence of *L. monocytogenes*. This study demonstrates that the current microbial safety and quality of REPFEDs is good, but that some improvements can still be made with regard to supplier selection, cleaning and disinfection, hygiene training and setting the shelf life duration. It also shows that producers should not rely on the reheating at consumer level to eliminate *L. monocytogenes*.

2.1 Introduction

The objective of this chapter is to provide an insight in the microbial safety and quality of the different types of REPFED products. The current microbial status of the products is evaluated at the production day and at the end of the shelf life, based on existing data from the participating companies. In addition, a systematic sampling of the production process was performed. Several studies have previously been performed in order to assess the microbial safety and quality of these cooked-chilled foods (Carlin *et al.*, 2004; Del Torre *et al.*, 2004; Mossel & Struijk, 1991; Rajkovic *et al.*, 2006). However, none of these studies systematically assessed the whole production process from start to finish for multiple microbial parameters in different REPFED companies. In this study samples were taken vertically throughout the production process, starting with raw materials and ending at the final products. To gain additional information on microbial quality and safety at the time of consumption, the end products were submitted to shelf life studies and a simulated reheating of the product at consumer level. Samples were analysed for multiple

microbiological parameters to create a comprehensive microbiological profile of the production process. Finally, as a case study, the growth potential of *Listeria monocytogenes* is determined in a specific REPFED (paella), including the recommended heat treatment for meal preparation at consumer level.

It must be noted that although this chapter examines the microbial safety of REPFEDs before and after shelf life, it does not take the variability of the consumer behaviour into account: e.g. temperature abuse, consumption after the end of shelf life or inadequate reheating. These factors will considerably affect the consumer exposure and will be discussed in chapter 6.

2.2 Materials and methods

2.2.1 Microbial safety of REPFEDs on the market based on historical data

Historical results of microbiological analyses of REPFED end products for *B. cereus*, *L. monocytogenes* and sulphite reducing Clostridia (SRC) were collected from five REPFED-producing companies. The companies took the samples in 2009 in the framework of their respective food safety management system. Various accredited laboratories analysed the samples and the results were reported back to the companies, which provided these data for analysis. The data were compared to the microbiological guide values established by the Laboratory of Food Microbiology and Food Preservation (LFMFP), for products on the day of production (T_0) and at the end of shelf life (T_{EoS}) (Table 2.3) (Uyttendaele *et al.*, 2010).

2.2.2 Microbiological assessment of the production process - New data

To cover the three different types of REPFEDs (See chapter 1, section 1.1.2), a sampling was performed in five REPFED-producing companies. Each company produced at least one of these types of REPFEDs.

2.2.2.1 Sampling locations

Both food products and food contact surfaces (e.g. machines and gloves) were sampled during production, from raw material through the production process until the end products. Seven groups of raw materials were included in the sampling plan (Table 2.1): (i) Raw meat and fish, (ii) raw vegetables and fruit, (iii) dry herbs and spices, (iv) ingredients heat treated by the supplier

with prior controlled heat treatment of 2 min 70°C or equivalent (e.g. pasteurised milk), (v) ingredients heat treated by the supplier without prior controlled heat treatment of 2 min 70°C or equivalent (e.g. blanched vegetables), (vi) dry pasta and rice and (vii) other powders and starches. A detailed overview of the sampling locations is shown in figure 2.1 and Table 2.1.

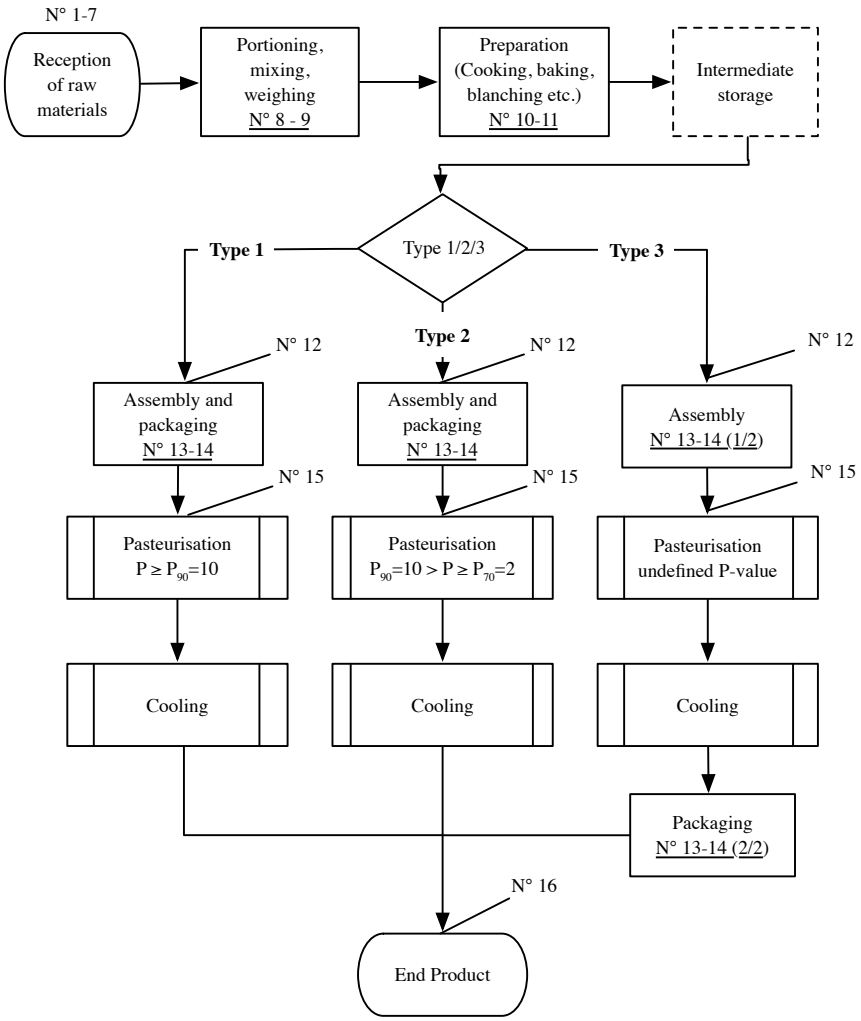


Figure 2.1: Generic flowchart of the REPFED production processes. "N°" indicated the different sampling locations as listed in table 2.1. Underlined sampling locations apply to contact surfaces or gloves. Sampling locations that are not underlined are product samples. P_{90}; pasteurisation value at 90°C, P_{70}: pasteurisation value at 70°C.

The sampling was performed on three sampling days, spread over a six-month period. On each sampling day, each group of raw materials was sampled once and all the other sampling locations were sampled three times. These three times corresponded with the start, middle and end of the production process. Therefore, the interval between sampling times (3-6 hours) depends on the length of the production time in a company (8-18 hours). Sampling frequencies are listed in Table 2.1.

Samples were analysed for three to five microbiological parameters (Table 2.1). Total psychrotrophic aerobic count (TPAC), aerobic spore count (ASC) and sulphite reducing Clostridia (SRC) were used as overall quality indicators for vegetative and spore forming organisms respectively. For the pathogenic microorganisms *L. monocytogenes* and *B. cereus* were selected since these are among the microorganisms of concern in REPFEDs (ACMSF (1992), EFSA (2005a); Hansen & Knochel (2001); Mackey & Bratchell (1989); Peck *et al.* (2008)). Although type 1 and 2 REPFEDs are processed with an in pack pasteurisation of at least 2 minutes 70°C, these products were also tested for *L. monocytogenes*, to get an insight in the need for a safe harbour pasteurisation step, in the contamination pressure from the raw materials and in the production environment.

Two types of sampling methods were used in this study (Table 2.1). Destructive sampling was used for raw materials (N° 1-7) and other food samples (N° 12, 15-16). Non-destructive sampling was used for food contact surfaces and gloves/hands of food handlers (N° 8-11, 13-14). For food samples a 100g sample was taken with a sterile spoon or tweezers and put aseptically in a sterile sampling-bag (TWIRL'EM, Labplas, Ste-Julie, QC, CA). Food contact surfaces and gloves/hands were swabbed using sterile cotton swabs (Rayon Swab, Novolab, Geraardsbergen, BE), which were pre-moistened in sterile peptone water (7 ml) for enumeration purposes or with demi-Fraser medium (7ml) for detection of *L. monocytogenes*. An area of 50 cm^2 was delimited using a sterile template and then swabbed, after which the swab was aseptically put back into its tube. Both product-samples and swabs were transported and stored cold ($\leq$ 4°C) and analysed within 24 hours. Methods used for enumeration are given in section 2.2.5.

2.2.2.2 Shelf life study and simulation of reheating by the consumer

To determine product safety and quality at the end of shelf life, end products were stored until the end of shelf life according to the guidelines by the EU Community Reference Lab (CRL) for *L. monocytogenes* (2008). The storage temperature was set at 8°C, the 75% percentile of data gathered from Belgian consumers (De Vriese *et al.*, 2005; EFSA, 2007; Vermeulen *et al.*, 2011).

For a shelf life up to 21 days (e.g. 18 days), the products were stored at 4°C for two thirds of the shelf life period to simulate internal storage and storage in display cabinets (e.g. 12 days) and one third of the shelf life at 8°C to simulate home storage (e.g. 6 days). For a shelf life longer than 21 days (e.g. 31 days), the product was stored at 4°C for a period of 7 days plus half of the remaining shelf life (e.g. (31-7)/2 =12 days, thus in total 7+12 =19 days at 4°C) and stored at 8°C for the remainder of the shelf life (e.g. 12 days). After storage one of the product samples was analysed cold and one sample was reheated before analysis. The product was reheated according to the instructions on the product label, using a microwave (Proline SM107, 700 W) or an electric oven (Samsung CM1929, 1850W), according to the procedure in section 2.2.3.

2.2.2.3 Data processing and interpretation of results

Results of the analysis were compared to a set of legal EU criteria (Anonymous, 2005, 2009). If there were no legal criteria, the microbiological guide values established by Laboratory of Food Microbiology and Food Preservation of the University of Ghent (LFMFP-UGent) were used for comparison (Uyttendaele *et al.*, 2010). For the end products these values are listed in table 2.2. Because there are no microbiological criteria available for intermediate products and food contact surfaces, the following guide values were used for pathogenic micro-organisms: (i) for intermediate products: $\leq$ 2 log CFU/g *B. cereus* and absence of *L. monocytogenes* in 25g. (ii) For food contact surfaces: $\leq$ 1.5 log CFU / 25cm^2 for *B. cereus* and absence of *L. monocytogenes* per 50cm^2. These guide values correspond to the limit of detection using standard methods for microbiological analysis. Because there are no legal criteria microbiological or guide values for TPAC, ASC and SRC on intermediate products or food contact materials, these results were compared to the arbitrarily chosen value of 6 log CFU/g for intermediate products and 3 log CFU/ 25cm^2 for food contact materials.

Table 2.1: Sampling locations with description of samples, microbiological parameters tested, sampling frequency and the total number of samples tested in the five companies.

N°	Description	Microbiological parameters[a]	Frequency [b]	nr. of samples
1	Raw meat or fish	TPAC, ASC, BC, LM, SRC	3 X 1	17
2	Raw vegetables or fruit	TPAC, ASC, BC, LM, SRC	3 X 1	14
3	Dry herbs and spices	ASC, BC, SRC	3 X 1	19
4	Ingredients with safe harbour	TPAC, ASC, BC, LM, SRC	3 X 1	16
5	Ingredients without safe harbour	TPAC, ASC, BC, LM, SRC	3 X 1	25
6	Dry pasta or rice	ASC, BC, SRC	3 X 1	18
7	Powders or starches	ASC, BC, SRC	3 X 1	14
8	Contact materials at raw material processing	TPAC, ASC, BC, LM	3 X 3	32
9	Hands/gloves at raw material processing	TPAC, ASC, BC, LM	3 X 3	18
10	Contact materials during preparation	TPAC, ASC, BC, LM	3 X 3	108
11	Hands/gloves during preparation	TPAC, ASC, BC, LM	3 X 3	36
12	Prepared product prior to packaging	TPAC, ASC, BC, LM, SRC	3 X 3	94
13	Contact materials during packaging	TPAC, ASC, BC, LM, SRC	3 X 3	86
14	Hands/gloves during packaging	TPAC, ASC, BC, LM	3 X 3	38
15	Packaged product prior to pasteurisation	TPAC, ASC, BC, LM, SRC	3 X 3	29
16	End product: day of production	TPAC, ASC, BC, LM, SRC	3 X 3	45
			Total:	609

(a) TPAC: Total psychrotrophic aerobic count; ASC: aerobic spore count; BC: *B. cereus*; LM: *L. monocytogenes*; SRC: sulphite reducing Clostridia; (b) nr. of sampling-days x nr. of samples per day

Table 2.2: Legal criteria (in bold) and microbiological guide values for selected microorganisms in pasteurised products at day of production (T_0) and end of shelf life (T_{EoS}) (Uyttendaele *et al.*, 2010). Values are shown in CFU/g. N.A.: not applicable.

	Type 1 and 2		Type 3	
Parameter	T_0	T_{EoS}	T_0	T_{EoS}
TPAC	10^4	10^6	10^4	10^{6a}
SRC	10^3	10^5	10^3	10^5
B. cereus	10^3	10^5	10^3	10^5
L. monocytogenes	N.A.	N.A.	**Absent in x g** [b] **Absent in 25 g** [c] **100 / g** [d]	**10^2**

(a) If the TPAC at the end of shelf life exceeds 10^6 CFU/g the product can only be rejected if it can be demonstrated that this does not concern lactic acid bacteria. It is recommended to check which TPAC are lactic acid bacteria. For lactic acid bacteria the recommended acceptable level is 10^7 CFU/g.

(b) Depends on growth potential - Depending on intrinsic and extrinsic product factors and the duration of the shelf life, these values should be adapted to guarantee that the maximum concentration on T_{EoS} (10^2 CFU/g) will not be exceeded.

(c) If the extent of growth of *L. monocytogenes*, during shelf life under prescribed storage conditions, cannot be demonstrated by the food business operator than the target level (Absence in 25 g) should be upheld.

(d) This target level is only valid if *L. monocytogenes* is unable to grow due to intrinsic and/or extrinsic factors and according to EU directive 2073/2005 (Anonymous, 2005) also for: (i) products with pH $\leq$ 4.4 or $a_w \leq 0.92$, (ii) products with pH $\leq$ 5.0 and $a_w \leq 0.94$, (iii) products with a shelf life shorter than 5 days and (iv) other product-categories, if scientific evidence supports this.

2.2.3 Characterisation of the heat treatment recommended at consumer level

Because there were no pre-existing data about the product temperature during reheating, 38 products were reheated according to the instructions provided by the manufacturer on the package. The heat treatment was simulated using a microwave (Proline SM107, 700 W) or an electric oven (Samsung CM1929, 1850W). For microwaveable products that required higher power output levels (more than 700 W), the recommended heating-time was increased to deliver the same energy (J) during reheating. For example, if the recommended heat treatment was 4 min at 800 W (192.0 kJ), the product was reheated for 4 min and 34 seconds at 700 W (191.8 kJ). During the heat treatment the temperature was measured every thirty seconds at the periphery and in the core of the product (Hanna instruments, HI 145-00, Ijselstein, NL). For each location (periphery/core) the P-value was calculated using equations 2.1 and 2.2: with t the time in min and $T(t)$ the tem-

perature in °C at time t. The z-value for *C. botulinum* (eq. 2.2) is temperature dependent (eq. 2.3).

$$P_{70} = \int_0^t 10^{\frac{T(t)-70}{7.5}} dt \tag{2.1}$$

$$P_{90} = \int_0^t 10^{\frac{T(t)-90}{z}} dt \tag{2.2}$$

$$z = \begin{cases} 7°C & \text{if T(t)} \leq 90°C; \\ 10°C & \text{if T(t)} > 90°C. \end{cases} \tag{2.3}$$

Since the target-organism during reheating is *L. monocytogenes*, the P_{70} was calculated (eq. 2.1). However, in some products the recorded temperature was sufficiently high to affect bacterial spores. Therefore, the P_{90}-value of these products was calculated using equation 2.2. If a product consisted of multiple components (e.g. meat and vegetables), the temperature and P-value were determined separately for each component.

2.2.4 Growth potential of *L. monocytogenes* in paella during shelf life and inactivation during heat treatment

The case of "paella", an example of a very heterogeneous REPFED (composed of meat, rice, chicken, vegetables) was chosen to assess the risk posed by *L. monocytogenes* after consumer storage and reheating. The paella in question, although subjected to heat treatment during production, is susceptible to post-processing recontamination during packaging and not all ingredients obtained a $P_{70} = 2$ or $P_{90} = 10$ during production (e.g. freshly added herbs, blanched vegetables). A challenge tests was performed according to the protocol described by the EU community reference lab for *L. monocytogenes* to determine the growth potential (δ) of *L. monocytogenes* during shelf life (EU CRL for *Listeria monocytogenes*, 2008). At the end of its shelf life, the contaminated paella was reheated according to the instructions of the manufacturer. This was done to determine the potential for survival of *L. monocytogenes* after heat treatment at consumer level. Three batches of paella, each consisting of fifteen packages, were purchased at a local supermarket. Nine of these fifteen packages were inoculated with *L. monocytogenes* and six packages were used as control samples and inoculated with sterile PPS (NaCl 8.5 g/l, peptone 1g/l (Oxoid)). In addition to these three batches, four extra samples were used to determine the temperature and pasteurisation value (P_{70}/ P_{90}) obtained in the chicken and rice component

when the product was reheated. Temperature measurements were performed according to the procedure described in section 2.2.3.

A cocktail of three *L. monocytogenes* strains: LMG 13305 (serotype 4b, soft cheese, clinical isolate) LMG 23194 (serotype 4b, soft cheese, food isolate) and LMG 23356 (serotype 4b, Jalisco cheese, food isolate) obtained from the Belgium Co-ordinated Collection of Microorganisms (BCCM, Ghent, BE) were used to perform the challenge tests as described by Vermeulen *et al.* (2011). In short, dilutions of the subculture of each of the three strains were mixed and 200 μl of the cocktail was dispersed across the surface of the paella to mimic contamination during packaging until an inoculum level of ±50 CFU/g was obtained. All fifteen products were packed under modified atmosphere consisting of a 50:50 mixture of N_2 and CO_2 (Airproducts, Vilvoorde, Belgium) using a Multivac C300 packaging machine (Haggenmuller, Wolfertschwenden, Germany) in combination with a gas-mixing device (WITT KM100-4MEM, Witten, Germany). The air concentration was checked on dummy packages using a Servomex Food Package Analyzer Series 400 (CISMA, Zoetermeer, The Netherlands). After packaging all samples (inoculated and control) were stored at 4°C (as recommended on the label) until the end of shelf life, which was 6 days after purchase.

The nine inoculated packages per batch were split in three groups: (i) Three of the inoculated packages were used to determine the initial inoculum level (T_0). (ii) Three were used to determine the concentration of *L. monocytogenes* at the end of shelf life (T_{EoS}) and (iii) three packages were used to check the presence of *L. monocytogenes* after heat treatment (microwave 4 min, 700 W). The six control samples, three at day 0 and three at the end of shelf life, were used for the determination of pH (SevenEasy pH, Mettler-Toledo GmbH, Schwerzenbach, Switzerland), a_w (a_w-kryometer AWK-20, NAGY messysteme GmbH, Gaüfelden, Germany), NaCl content (Mohr method with K_2CrO_4 (UN3288, Merck KGaA, Damstadt, Germany) and $AgNO_3$ (80289927, Merck)), headspace composition (PBI Dansensor type Checkmate 9900 O2/CO2 (Ringsted, Denmark)), total psychrotrophic aerobic count (TPAC), lactic acid bacteria (LAB), yeasts and moulds (Y&M) and *B. cereus*. Methods used for enumeration are given in section 2.2.5.

2.2.5 Microbiological methods for enumeration

Standardised methods for enumeration were:

- TPAC: ISO 6222: 1999, plating on plate count agar (PCA, Oxoid, Basingstoke, UK) and 72-120h incubation at 22°C.

- *B. cereus*: ISO 7932: 2004 , plating on manitol egg yolk polymyxine agar (Oxoid) and incubating 24h at 30°C, confirmation on trypton soy agar with 5% Sheep Blood (BD, Erembodegem, BE).
- SRC: AFNOR (1996) XP V 08-61, plating on tryptose sulphite cycloserine agar (Oxoid) and anaerobic incubation at 37°C for 20h.
- LAB: plating on de Man Rogosa Sharpe agar (MRS, Oxoid) with 1.4 g/l sorbic acid (min 99%, Sigma-Aldrich) and a top layer. The plates were incubated for 72 h at 30 °C.
- ASC: method recommended by the Belgian national reference lab (1998): SP-VG M008, which consists of heating the primary dilution for 10 minutes at 80°C followed by plating on PCA and incubation for 72h at 37°C.
- Yeasts and moulds: plating on Yeast Glucose Chloramphenicol agar (YGC, Bio-Rad, Marnes-La-Coquette, France) and incubated for 72 h at 30 °C.
- *L. monocytogenes*: For detection in food samples a 25g subsample was taken from each food sample and pre-enriched in 225ml of demi-fraser medium. Pre-enrichment of swabs was performed in the tubes filled with demi-fraser. Detection of was carried out using the VidasLMO2 method (Biomérieux, Marcy l'Etoile, FR) an AFNOR validated (n° BIO-12/11-03/04) rapid Enzyme-Linked Fluorescent Assay. The enumeration for *L. monocytogenes*, using ISO 11290-2: 1998, (plating on ALOA (Biolife, Milan, IT) and incubating 24h at 37°C) was only performed for food samples and only if VidasLMO2 detection in 25g was positive. A list of the parameters that was analysed per sampling location is provided in Table 2.1.

2.3 Results and discussion

2.3.1 Microbial safety of REPFEDs on the market based on historical data

The collection of historical data resulted in data for 1530 different food products. Because most samples were analysed for multiple parameters at multiple times (T_0 and T_{EoS}), this resulted in a total of 3618 analyses. Table 2.3 shows an overview of these historical data per parameter and per time of analysis. Only 2.94 % of the products were positive for one of the three pathogenic microorganisms and as little as 2 out of 1530 (0.13%) samples exceeded the acceptable levels (table 2.2) Moreover, in both cases the products (potato gratin and meatballs in tomato sauce) were subjected to a considerable period (4-12h) of temperature abuse (room temperature) on

the day of production. These results indicate that the current microbial safety of the examined REPFEDs is satisfactory.

Table 2.3: Results of a screening of commercial REPFEDs, expressed as the amount of positive samples and the amount of samples that exceeded legal guidelines and criteria (Table 2.2). Percentages are expressed as part of the number of analyses per time and per parameter

Number of	Day of production (n= 706)			End of Shelf life (n=2912)			Total nr. of
Analyses	LMO[a]	BC[b]	SRC[c]	LMO	BC	SRC	products
Done	79	369	258	1,117	930	865	1,530
Positive	0	15 (4.07%)	5 (1.94%)	0	19 (2.04%)	6 (0.69%)	45 (2.94%)
≥ acceptable levels	0	2 (0.54%)	0	0	0	0	2 (0.13%)

(a) *L. monocytogenes*; (b) *B. cereus*; (c) sulphite reducing clostridia

2.3.2 Assessment of the production process per sampling location

In tables 2.7 to 2.9 (p.65-67) detailed microbiological results for the three types of REPFED production processes are given. Type 1 products are in-pack pasteurised for at least 10 minutes 90°C. Type 2 products are in-pack pasteurised for at least 2 minutes at 70°C and type 3 products are pasteurised in an open package or repacked after in-pack pasteurisation (see chapter 1 for a more detailed discussion). The results were compared to the legal EU criteria (Anonymous, 2005) and the microbiological guide values established by the LFMFP of the University of Ghent (Table 2.2) (Uyttendaele *et al.*, 2010).

2.3.2.1 Raw materials

All but one of the raw material samples complied with the microbiological guide values for ASC and SRC, one sample of herbs and spices contained high counts of SRC (4.2 log CFU/g). Non-compliances in raw materials (N° 1-7) were mainly linked to three parameters: *B. cereus*, *L. monocytogenes* and TPAC. *B. cereus* was enumerated in 3 of 19 samples of dried herbs and spices in high numbers (> 10^4CFU/g). The implicated raw materials were basil, fennel and oregano. *B. cereus* spores are a common contamination on dried herbs and spices (Konuma *et al.*, 1988; Powers *et al.*, 1976). For TPAC, 6 of 72 samples exceeded microbiological guide values (> 10^6 log CFU/g), half of them on raw meat and fish (N° 1). These TPAC levels are not a direct food safety issue, but an indication of the insufficient microbial quality of specific raw materials.

Higher contaminated raw materials increase the pressure on the downstream process to deliver food products with low contamination levels (Luning *et al.*, 2011).

The third microorganism of concern in raw materials was *L. monocytogenes*. While all of the samples were below the limit of quantification (<10 CFU/g), *L. monocytogenes* was detected (presence in 25g) in 9 out of 72 raw material samples. Five out of seventeen samples of raw meat or fish (29.4%) and 4 out of 25 (16.0%) samples of ingredients without prior controlled heat treatment of 2 min 70°C or equivalent (N° 5) were found positive. Among this last type of ingredients that contained *L. monocytogenes* were: grilled meatballs, pre-cooked hamburgers and minced pre-cooked lamb meat. These results are not unexpected considering the occasional prevalence of *L. monocytogenes* as reported for raw and cooked meat, poultry and fishery products (Farber & Peterkin, 1991; FASFC, 2006; ICMSF, 2005; Jay, 1996; Johnson *et al.*, 1990; Lo Nostro *et al.*, 2010; Ochiai *et al.*, 2010).

The microbial quality and safety of the sampled raw materials and ingredients confirm that these products are a potential source of microbial hazards entering the production process: *B. cereus* on dried herbs, *L. monocytogenes* on raw meat and fish and on pre-cooked meat products (without a prior and validated heat treatment of 2 min 70°C or equivalent) and elevated TPAC on raw meat and fish. The microbial quality and safety of raw materials may be improved by supplier selection, the setting of purchase specifications and compliance testing (Luning *et al.*, 2008).

2.3.2.2 Intermediate products

L. monocytogenes was detected in 9.8% (12 of 123) of the intermediate product samples (N° 12 and 15). The prevalence varied between companies, ranging from 0% to 21.7% positive samples. In two companies the contamination may be linked to the presence of a *L. monocytogenes* contamination in the production environment or in raw materials. In the first company *L. monocytogenes* was present on a packaging installation (see section 2.3.2.3) and on the product (a type 3 pasta product) sampled during packaging. Contamination via the production environment by *L. monocytogenes* has also been previously reported and can be reduced by improved cleaning and disinfection (Lunden *et al.*, 2002; Lyytikainen *et al.*, 2000; Reij *et al.*, 2004). In the second company *L. monocytogenes* was present in a raw material and remained present in the prepared product (meatballs in onion sauce) until it was subjected to pasteurisation (90°C for 10 minutes), after pasteurisation *L. monocytogenes* was no longer detected.

B. cereus could be enumerated ($\geq 10^2$ CFU/g) in five of the 123 samples: in one sample of mashed potatoes in a type 1 production process (P_{90}=10) and in four samples in a type 2 produc-

tion process (P_{70}=2): minced meat preparation, chicken with curry sauce and twice in bolognaise sauce. The maximum *B. cereus* count was 3.8 log CFU/g (type 1 mashed potatoes). Rajkovic et al. (2006) reported a similar contamination in a type 2 potato puree. Which was not unexpected as *B. cereus* is able to survive the heat treatment that is applied in these type 2 processes (70°C for 2 min). It is important that no growth or *B. cereus* occurs in the intermediate products during processing prior to pasteurisation. The growth of *B. cereus* to elevated levels (10^5 log CFU/g) permits the formation of the heat stable emetic toxin (cereulide), which is not inactivated during pasteurisation (Rajkovic *et al.*, 2008).

A limited number (n=5) of intermediate products exceeded the microbiological guide values for TPAC (10^6 log CFU/g). These products were found in different types and different companies: vegetable and meat mash (n=2), cold cooked pasta (n=1) and chicken ragout (n=2). The majority (4 of 5) of products exceeding the microbiological guide values were found at the middle or end of the production process and not at the start of production process. This indicates that growth during production may be a bottleneck in the current food safety management system.

2.3.2.3 Food contact surfaces and gloves

Samples were taken of 226 food contact surfaces (N° 8, 10 and 13) and 92 pairs of gloves of employees in the processing area (N° 9, 11 and 14). None of the sampled gloves were positive for *L. monocytogenes*. However, *L. monocytogenes* was detected in four of 226 environmental food contact surface samples (1.8%) but all counts were below the quantification limit (< 1.5 log CFU / 25cm^2). Three of the four positive samples were found in one type 3 company and all three were detected in samples from the same day. The positive samples were detected at the cooling and dispensing of a freshly cooked pasta-product. The most likely source of this contamination was the processing equipment for cooling and dispersing, because *L. monocytogenes* was not found on the pasta product directly after cooking and before cooling. The samples of the processing equipment that were positive for *L. monocytogenes* were taken at the middle (n=1) and the end (n=2) of the production day.

B. cereus was found on both food contact surfaces (6 of 226) and gloves (2 of 92). Five out of the six *B. cereus* contaminations on food contact surfaces occurred in leftovers of sauce on sauce-dispensers, which were used for an extended period of time without cleaning. The contamination ranged between 1.8 and 2.7 log CFU / 25cm^2. The sixth positive sample was taken of the ceiling above the opening of a cooking-kettle (2.1 log CFU / 25cm^2). Presence of *B. cereus* on these locations can lead to spot contaminations in the intermediate product. Since spot contaminations are difficult to predict, problem awareness and appropriate control measures in the

food safety management system (FSMS) are recommended to tackle this problem. A solution may be more frequent intermediate cleaning and disinfection of these locations to avoid growth and subsequent recontamination. Tompkin (2002) stated that more frequent cleaning may not be effective and even counterproductive because a wet environment should be avoided during production. However, all the production environments sampled in this study were moist or wet during production. Yet it must be noted that water droplets may help spread the contamination (den Aantrekker *et al.*, 2003).

The overall microbial quality of food contact surfaces and gloves was not satisfactory. In total 39.8% of surface samples contained elevated counts of TPAC (> 10^3 CFU/g). It has to be noted that there are currently no widely accepted microbiological guide values for environmental samples such as food contact surfaces and gloves and that all samples were taken during production when contamination of gloves with TPAC from contact with raw or intermediate products is likely. Trend monitoring is recommended to determine appropriate microbiological guide values for TPAC on food contact surfaces and gloves. This monitoring should take into account the various processes that are performed at different locations and the direct or indirect contact with foods as well as the type of foods handled (Lahou *et al.*, 2012). The elevated TPAC values are not necessarily a food safety problem, they are an indication of the general hygiene and GMP during production (Lasta *et al.*, 1992).

For ASC a small number of samples (5 of 315) exceeded the guide values. The locations of these samples were similar to those for *B. cereus*: inside a kettle, on the ceiling above the opening of a kettle, in a collector for mashed potatoes, on a conveyer belt, on a sauce dispenser and on the underside of a electricity box above a conveyer belt.

The frequent contamination of food contact surfaces and gloves with TPAC and the sporadic contamination with *B. cereus* and *L. monocytogenes* increases the pressure on the food safety management system to guarantee the microbial safety and quality of the end products. It also points out the need for continuous attention to control measures such as improved hand hygiene and frequent cleaning and disinfection.

2.3.2.4 End products

Results for the end products at day of production (n=45) are shown in tables 2.7 - 2.9 (N° 16) (p. 65-67), results of the shelf life study (n=90) in table 2.10 (p.68). The shelf life of REPFEDs in the present study ranged from 10 to 45 days. The microbial quality of end products of four companies was satisfactory. Only the products of one company regularly contained high counts

for TPAC, both on the day of production and at the end of shelf life (up to 8.6 log CFU/g). These results were linked to the company's production process, which consisted of a pasteurisation process (P_{90}=10) in larger volumes, followed by a repackaging in smaller volumes in a high-care zone. Because the food contact surfaces in the high-care zone contained elevated TPAC, products were at risk of being recontaminated during packaging (Reij *et al.*, 2004).

The microbial safety of all the end products tested (n=135) was satisfactory. Of the two pathogens tested in the present study only *B. cereus* was found in end products (n=9), and only in type 2 end products. The respective counts never exceeded 2.7 and 1.6 log CFU/g and the counts did not increase during shelf life (storage at max. 8°C), which indicates that it were most likely mesophilic *B. cereus* strains. *B. cereus* can survive the mild pasteurisation process at 70°C, because the process is designed to eliminate *L. monocytogenes* and the temperature does not exceed 80°C (Carlin *et al.*, 2000a). *L. monocytogenes* was not detected (in 25g) in any of the 135 end products sampled, which indicates that the pasteurisation processes (type 1 and 2) and/or hygiene measurements taken to prevent recontamination are effective (type 3). Although it was unlikely that *L. monocytogenes* would be present on a type 1 or 2 product, the samples were still analysed to illustrate the importance of the heat treatment in the production process.

For TPAC, 16 of the 45 end products exceeded the acceptable levels at the end of shelf life, even after storage under refrigeration (Table 2.2). However, in 13 of 16 of these products reheating the end product according to instructions as mentioned on the consumer package reduced the TPAC to acceptable levels ($< 10^6$ CFU/g) (Table 2.10). After heat treatment only 3 in 45 samples exceeded the microbiological guide values for TPAC. These elevated TPAC may indicate a relatively long shelf life given for these specific REPFED products. It is recommended to re-evaluate this shelf life to maintain a satisfactory microbial quality throughout the shelf life. In addition it may help to validate and verify the heat treatment at consumer level since this can be considered an additional hurdle for pathogenic microorganisms.

2.3.3 Characterisation of the heat treatment recommended at consumer level

Because reheating at consumer level can reduce the risk posed by certain pathogens and *L. monocytogenes* in particular, 38 products (11 type 1, 10 type 2 and 17 type 3) were reheated according to the instructions on the label. During reheating, temperatures were monitored and the P-value was calculated. Seven products consisted of two components and for each of these components the temperature was monitored separately.

Table 2.4: Maximum measured temperatures (T_{max}) and calculated P_{70} or P_{90}-values for selected cooked-chilled foods during simulated reheating at consumer level. For multicomponent meals (e.g. meat and vegetables) each component was measured separately. The measured component is indicated in bold in the product description. Unless otherwise mentioned, products were reheated using a microwave oven.

Product description	Type	Periphery		Core		
		P_{70} (min)	**T_{max}** (°C)	**P_{70}** (min)	**T_{max} (°C)** (°C)	**$P_{70} \geq 2$**
Carrot and potato mash	1	0.18	71.2	1.19[a]	88	-
Carrot-potato mash & sausage	1	0.02	59.4	0.17	68	-
Carrot-potato mash & **sausage**	1	0.004	47.6	-	-	-
Leek and potato mash	1	0.25	71.5	2.7	93.1	-
Spinach mash	1	0.41	73.1	1.98	91.4	-
Mashed potatoes	1	7.16	80.3	0.003	57	-
Pork chops	1	5.00[a]	93	1.30	89.8	yes
Ratatouille	1	5.6	89.7	15.5	96.9	yes
Belgian Endives & potatoes	1	6.1	85.4	68.2	79.2	yes
Braised carrots	1	7.7	93.4	20.8	96	yes
Cow's tongue in madeira sauce	1	7.9	95.8	4.9	95	yes
Mashed potatoes	1	7.7	77.4	1.5	74.4	-
Hachee parmentier	2	0.002	47.6	0.082	64.3	-
Fish parmentier	2	0.0008	44.3	0.0002	49	-
Mashed potatoes	2	0.0175	61.7	0.0008	52.2	-
Meatballs in tomato sauce	2	0.00027	48.2	0.00078	52	-
Chicken & **rice** in curry sauce (n°1)	2	1.28	74.9	21.8	86.5	-
Chicken & rice in curry sauce (n°1)	2	0.0033	55.7	0.00037	48	-
Chicken & **rice** in curry sauce (n°2)	2	1.52	77.3	3.1	80.4	-
Chicken & rice in curry sauce (n°2)	2	0.0012	52	0.0003	48.1	-
Spaghetti carbonara	2	0	27	0	31.3	-
Spaghetti **bolognese**	2	0.004	57.8	0.01	50.5	-
Spaghetti bolognese	2	21.6	87.3	5.41	95.1	-
Tagliatelle **carbonara**	2	0.0039	48.2	0.0005	41.5	-
Tagliatelle carbonara	2	12.6	83.2	7.13	84.7	-
Salmon and **rice**	2	0.055	66.3	1.99	79.9	-

Continued on next page

Table 2.4 – continued from previous page

Product description	Type	Periphery		Core		
		$\mathbf{P_{70}}$ (min)	$\mathbf{T_{max}}$ (°C)	$\mathbf{P_{70}}$ (min)	$\mathbf{T_{max}}$ (°C)	$\mathbf{P_{70} \geq 2}$
Salmon and rice	2	0.0005	50	0.0005	39.2	-
Cannelloni (electric oven)	3	<u>2.3</u>	88.7	3.1	74.2	yes
Tagliatelle carbonara	3	20	80.5	0.01	53.8	-
Macaroni with ham and cheese	3	11.6	79.5	0.07	66.7	-
Tagliatelle carbonara	3	1.43	74.3	0.02	61.3	-
Tagliatelle with salmon	3	0.85	72.5	0.01	59.1	-
Spaghetti bolognese	3	0.08	72.6	0	53.5	-
Penne with vegetables	3	2.43	76.4	0.01	60.5	-
Tagliatelle carbonara	3	1.07	74.7	0	56	-
Tagliatelle with salmon	3	0.15	65.8	0.04	61.1	-
Veal stew & mashed potatoes	3	0.11	64.7	0.02	59.3	-
Veal stew & **mashed potatoes**	3	0.35	70.2	0	53	-
Spaghetti bolognese (1)	3	0.38	70.8	0.01	55.5	-
Spaghetti bolognese (2)	3	0.16	65.3	0.01	57.8	-
Spaghetti bolognese (3)	3	0.01	59.3	0	58.2	-
Ham rolls with Belgian endives (1)	3	0.13	64.4	0.02	55.3	-
Ham rolls with Belgian endives (2)	3	1.19	71.3	0.01	54.5	-
Ham rolls with Belgian endives (3)	3	0.06	62.7	0	51.5	-
Schnitzel	3	28.6	88.7	3.1	74.2	yes

(a): underlined P-values are P_{90}-values (eq. 2.2) instead of P_{70}-values (eq. 2.1)

The results of the reheating trials (table 2.4) show that only 7 of the 38 tested products received a P_{70}-value equal to or greater than 2 min at 70°C ($P_{70} \geq 2$ min) and could thus be catalogued as Ready-To-Heat (RTH). Of these 7 products, 5 were type 1 products and 2 were type 3 products. Thus of the type 1 products, 45.5% (5/11) obtained a $P_{70} \geq 2$, while this was only 11.8% (3/17) for type 3 and 0% for type 2 products. It has to be noted that both type 3 products that obtained a $P_{70} \geq 2$, were products with a simple structure (cannelloni and schnitzel). All other type 3 products were mixed-structure meals (e.g. pasta and sauce). Not only did more type 1 products obtain the targeted P-value, but the obtained P-values were also substantially higher for type 1 products than those for type 2 or 3 products. Four of the five type 1 products that received an adequate heat treatment, received a treatment higher than P_{90}=1, while this was not the case in the other product-types. It is surprising that particularly type 1 products achieve a P_{70} of 2 min during

reheating. These products have had the most severe heat treatment during processing and are not prone to recontamination and thus do not need to be reheated to eliminate *L. monocytogenes*. However, they are more likely to reach adequate temperatures and P-values during reheating. This difference can be partially explained by the difference in packaging. The type 1 products that obtained high P-values were packed in plastic pouches while type 2 and 3 products were mostly packed in trays. During reheating the bags retained more steam than the trays, which in turn increased the temperature in the bag. This illustrates the importance of packaging in the reheating step.

In addition to the seven products that received an adequate heat treatment (P_{70}=2) for the entire product, there were also several products in which the periphery (n=5), the core (n=4) or one of the components (n=2) received a $P_{70} \geq 2$ while other regions or components of the product did not. This was most clear in two type 2 products: chicken with rice in curry sauce and tagliatelle carbonara. While the starch components (rice and pasta) reached temperatures between 74.9°C and 95.1°C, the chicken or sauce did not exceed 57.8°C. In some cases, the temperature was higher at the periphery than at the core of the product, leading to a very heterogeneous heat profiles in the food product. These differences demonstrate that any reheating-process has to be validated for all spots and all components of a product. In addition, these results are in contradiction with the statement made by Cronin & Wilkinson (2009). They stated that consumer heating of cooked-chilled foods generally kills any vegetative *B. cereus*. However given the very low temperatures obtained during most of the reheating trials, this is highly unlikely. And this is without taking consumer behaviour into account.

2.3.4 Growth potential of *L. monocytogenes* in paella during the shelf life and inactivation during the heat treatment at consumer level

Three batches of a ready-to-heat type paella were inoculated with *L. monocytogenes* to assess the growth potential of this pathogen (EU CRL for *Listeria monocytogenes*, 2008). Results are shown in Table 2.5. The a_w, pH and NaCl-content of the products were well within the limits to enable growth of *L. monocytogenes* (ICMSF, 1996). Results show an acceptable microbial quality of the paella. The second and third batch contained elevated concentrations of LAB (±3.5 log CFU/g) and only the second batch contained low concentrations of *B. cereus* (max. 2.48 log CFU/g). The growth potential (δ) of *L. monocytogenes* at 4°C was determined at 0.63 log CFU/g, which means that the product can support the growth of *L. monocytogenes* even at low temperatures (4°C). According to EU regulation 2073/2005 (Anonymous, 2005) the maximal count at the end of shelf life cannot exceed 100 CFU/g. Thus a theoretical maximum tolerable

Table 2.5: Results of a *L. monocytogenes* challenge test on a paella REPFED (type 3) including reheating at consumer level. Products were inoculated after purchase (50-100 CFU/g) and stored at 4°C.

Batch	Batch - properties	Day of purchase					End of shelf life (EoS) Cold					EoS heated	Δ^f	δ^g
		TPAC[a]	LAB[b]	Y&M[c]	BC[d]	LMO[e]	TPAC	LAB	Y&M	BC	LMO	LMO		
	a_w: 0.987 ± 0.0015													
	pH: 6.51 ± 0.03	2.77	< 1	< 1	< 1	1.3	4.66	< 1	< 1	1.7	2.11	A[h]		
1	NaCl: 1.47 ± 0.07% (w/w)	2.72	< 1	< 1	< 1	1.7	< 3	< 1	< 1	< 1	2.11	P	0.63	
	MAP:0.97 ± 0.93 % O_2	2.82	< 1	< 1	< 1	1.48	3.3	< 1	< 1	< 1	1.95	P		
	28.13 ± 2.32 % CO_2													
	a_w: 0.987 ± 0.0005													
	pH: 6.45 ± 0.03	3.3	< 1	< 1	< 1	1.95	6.97	3.11	< 1	1.6	2.48	P		
2	NaCl: 1.52 ± 0.03% (w/w)	3.78	< 1	< 1	< 1	2	7.61	3.69	< 1	2.18	2.45	P	0.5	0.63
	MAP:0.21 ± 0.23 % O_2	4	< 1	< 1	< 1	1.7	7.32	3.4	< 1	1.3	2.36	A		
	29.00 ± 3.77 % CO_2													
	a_w:0.985±0.007													
	pH: 6.44 ± 0.03	2.74	< 1	< 1	< 1	1.48	4	3.69	< 1	< 1	1.85	P		
3	NaCl: 1.65 ± 0.06% (w/w)	2.68	< 1	< 1	< 1	1.48	4.47	4.15	< 1	< 1	2.2	A	0.37	
	MAP:0.30 ± 0.22 % O_2	2.61	< 1	< 1	< 1	1.6	4.04	2.41	< 1	< 1	1.3	A		
	29.97 ± 3.62 % CO_2													

(a) total psychrotrophic aerobic count; (b) lactic acid bacteria; (c) Yeasts and moulds; (d) *B. cereus*; (e) *L. monocytogenes*; (f) Δ= Median EoS (cold) - Median day of purchase; (g) δ: growth potential for *L. monocytogenes* (= max of Δ); (h) A/P: Absent/Present in 25g of product

concentration of 10 CFU/g just after production is acceptable, taking the possible growth of *L. monocytogenes* during shelf life into account. However, this does not consider the actual storage temperature and it is recommended to strive for absence in 25g.

The temperatures and P-values obtained during reheating of the paella samples were highly variable (Table 2.6). Two of the four reheated samples obtained a P_{70} of 2 min throughout the entire product and could be categorised as ready-to-heat, while the other two samples did not obtain a P_{70} of 2 min and can be categorised as ready-to-reheat. However, the maximal temperatures reached in the rice during reheating ranged from 68.0°C (P_{70} = 0.2 min) to 90.3°C (P_{70} = 83.0 min). This broad temperature range means that the theoretical reduction of *L. monocytogenes* ranged from 0.6D to 249D (based on 2 min at 70°C for a 6D reduction). The variation in P-values explains why *L. monocytogenes* was still present (in 25g) in five out of nine product samples after reheating and demonstrates the importance of validating the reheating as recommended to the consumer. Given these results, the paella should be categorised as ready-to-reheat and the producer should not take the reheating at consumer level into account when setting criteria for *L. monocytogenes*.

Table 2.6: Maximum temperatures and P_{70}-values measured in two components (rice and chicken) of paella during microwave reheating as advised on the label (4 min 700 W).

	Rice		Chicken	
Sample[a]	T_{max} (°C)	P_{70} (min)	T_{max} (°C)	P_{70} (min)
1	72	0.3	73.6	1.2
2	74.2	2.1	77.4	5.7
3	90.3	83	83.8	73.7
4	68.4	0.2	79.6	4.2

[a] sample numbers are not related to batch numbers used in table 2.5

2.4 Conclusions

The sampling study presented in this chapter provides insight in the location and variability of the microbial contamination in the production process of REPFEDs and in the quality and safety of REPFEDs put on the market. From the results of these microbiological analyses some points of attention can be raised. Although a heat treatment is involved in the production of cooked-chilled foods, the potential contamination with *L. monocytogenes* of raw fresh meat, fish and pre-cooked meat products used as raw materials may introduce the pathogen in the production environment with subsequent potential for post-contamination (i.e. type 3 products). The overall microbial

load on food contact surfaces in the production environment (both hands/gloves of personnel and equipment), which may reach high numbers during processing, has to be monitored. This contamination may lead to opportunities for recontamination or spot contamination, which in turn lead to increased levels of TPAC or *B. cereus* on intermediate or end products. This is of particular importance for type 3 REPFEDs. The initial quality of the product will affect the quality of the product at the end of shelf life and in particular with long shelf lives, this may lead to unacceptable quality at the time of consumption. From these microbiological results several recommendations were deduced, which should be included when establishing a well-elaborated food safety management system: (i) Supplier selection and monitoring of the microbial quality of incoming raw materials. (ii) Cleaning and disinfection of production environment in order to prevent recontamination of intermediate products during processing. (iii) Hygiene training of personnel in order to control contamination levels of hands or gloves and finally (iv) proper shelf life validation under reasonable foreseen conditions of time and temperature of storage at the consumer.

Challenge testing confirmed that *L. monocytogenes* could grow in a sample product and could survive reheating at consumer level. Given the recorded amount of variability in temperature and P_{70}-value it is clear that reheating by the consumer cannot be considered an effective hurdle to assure food safety with respect to *L. monocytogenes* or any other heat-resistant pathogenic microorganisms. Furthermore, as mentioned in the introduction, this study does not take the actual consumer behaviour in to account. Products were stored (4°C) and reheated according to the instructions on the label. For Belgium it is known that the 75th percentile of the refrigerator temperature is 8°C (Vermeulen *et al.*, 2011), which will increase the growth rate of *L. monocytogenes*. Similarly it is likely that not all consumers will respect the reheating instructions (chapter 6). Therefore, the reheating at the consumer level should not be taken into account when setting a maximum tolerable concentration for *L. monocytogenes* on day 0. Despite these potential risks, the current microbial food safety of the tested REPFEDs is satisfactory.

As shown in this chapter, REPFEDs are a complex food group, containing a wide diversity of raw materials and ingredients, production processes, shelf life conditions and instructions for use by consumers. To effectively assess the microbiological risks related to REPFEDs more comprehensive information is required on consumer behaviour, more specifically on the time between purchase and consumption and on the amount of products that is consumed after the end of shelf life. This information is discussed at length in chapter 6.

Table 2.7: Detailed results (minimum-maximum counts) for **type 1** REPFEDs based on compiled data of multiple companies. Counts are shown in log CFU/g for product samples (N° 1-7, 12, 15-16) and log CFU/25 cm^2 for environmental or contact surface samples (N° 8-11, 13-14). Results for *L. monocytogenes* relate to presence/absence testing. Numbers between brackets in bold indicate the number of samples that exceeded the legal criteria or microbial guide values. Numbers between brackets but not in bold indicates presence or above detection limit but **compliance** to the legal criteria and guide values.

N°	Sample description	n	Quality indicators			Pathogenic microorganisms	
		231	TPAC[a]	ASC[b]	SRC[c]	LMO[d]	*B. cereus*
1	Raw meat or fish	5	2.7 - 4.6	<1 - <3	<1	P[e] (3/5)	<2 - 2.5
2	Raw vegetables or fruit	5	<4 - 8.2 **(1/5)**	<1 - 3.0	<1 - 1.0	A[e]	<2
3	Dry herbs and spices	7	-	<2 - 5.1	<1 - 2.6	-	<2 - 5.3 **(1/7)**
4	Raw materials with safe harbour	6	<2 - 4.7	<1 - 4.3	<1	A	<2
5	Raw materials without safe harbour	10	<2 - 6.7	<1 - 3.3	<	P (3/10)	<2
6	Dry pasta or rice	5	-	<1 - 4.7	<1	-	<2
7	Powders or starches	4	-	<2 - 3.9	<1	-	<2
8	Contact materials at raw material processing	11	<0.5 - 4.3 **(7/11)**	<0.5 - 1.5	-	A	<1.5 - 1.5
9	Hands/gloves at raw material processing	11	<0.5 - 5.3 **(8/11)**	<0.5	-	A	<1.5 - 1.5
10	Contact materials during preparation	37	<0.5 - 5.4 **(18/37)**	<0.5 - 3.1 **(1/37)**	-	A	<1.5 - 2.1 **(1/37)**
11	Hands/gloves during preparation	15	<0.5 - 7.1 **(9/15)**	<0.5 - 4.5 **(1/15)**	-	A	<1.5 - 3.3 **(2/15)**
12	Prepared product prior to packaging	28	1.0 - 6.1 **(1/28)**	<1 - 4.3	<1.0 - 2.6	P **(4/28)**	<2 - 2.0
13	Contact materials during packaging	35	2.0 - 6.3 **(27/35)**	<0.5 - 4.2 **(1/35)**	<0.5 - 2.4	P **(1/35)**	<1.5 - 1.5
14	Hands/gloves during packaging	16	1.7 - 6.8 **(10/16)**	<0.5 - 2.4	-	A	<1.5
15	Packaged product prior to pasteurisation	18	<2 - 7.0 **(1/18)**	<1 - 3.4	<1	P **(4/18)**	<2 - 3.8 **(1/18)**
16	End product: day of production	18	<2 - 3.1	<1 - 2.3	<1	A	<2

(a) TPAC: total psychrotrophic aerobic count; (b) ASC: aerobic spore count;(c) SRC: sulphite reducing Clostridia;

(d) LMO: *L. monocytogenes*; (e) A/P: Absent/Present in 25g or on 50cm^2

Table 2.8: Detailed results (minimum-maximum counts) for **type 2** REPFEDs based on compiled data of multiple companies. Counts are shown in log CFU/g for product samples (N° 1-7, 12, 15-16) and log CFU/25 cm^2 for environmental or contact surface samples (N° 8-11, 13-14). Results for *L. monocytogenes* relate to presence/absence testing. Numbers between brackets in bold indicate the number of samples that exceeded the legal criteria or microbial guide values. Numbers between brackets but not in bold indicates presence or above detection limit but compliance to the legal criteria and guide values.

N°	Sample description	n	Quality indicators			Pathogenic microorganisms	
		134	TPAC[a]	ASC[b]	SRC[c]	LMO[d]	*B. cereus*
1	Raw meat or fish	5	3.7 - 7.8**(2/5)**	< 1	< 1 - 1.7	A[e]	< 2
2	Raw vegetables or fruit	3	2.9 - 3.7	< 1 - 3.9	< 1	A	< 2
3	Dry herbs and spices	4	-	< 2 - <3	< 1 - 4.2 **(1/4)**	-	< 2
4	Raw materials with safe harbour	4	< 2 - 4.4	< 1 - < 2	< 1	A	< 2
5	Raw materials without safe harbour	3	2.8 - 4.6	< 1 - 3.7	< 1 - 1.0	P[e] (1/3)	< 2
6	Dry pasta or rice	5	-	< 1 - 3.0	< 1	-	< 2 - 3.5
7	Powders or starches	3	-	< 2 - <3	< 1 - 2.0	-	< 2
8	Contact materials at raw material processing	9	< 0.5 - 4.3 **(1/9)**	< 0.5 - 2.9	-	A	< 1.5
9	Hands/gloves at raw material processing	3	1.0 - 2.5	< 0.5	-	A	< 1.5
10	Contact materials during preparation	29	< 0.5 - 5.5 **(14/29)**	< 0.5 - 3.7 **(1/29)**	-	A	< 1.5 - 1.5
11	Hands/gloves during preparation	1	2.5	-	-	A	< 1.5
12	Prepared product prior to packaging	24	< 2 - 6.2 **(1/24)**	< 1 - 3.3	< 1 - 3.0	P **(2/24)**	< 2 - 2.5 **(1/24)**
13	Contact materials during packaging	17	< 0.5 - 5.0 **(6/17)**	< 0.5 - 3.4 **(1/17)**	< 0.5	A	< 1.5 - 2.5 **(5/17)**
14	Hands/gloves during packaging	6	0.5 - 4.4 **(3/6)**	< 0.5 - 3.8 **(2/6)**	-	A	< 1.5
15	Packaged product prior to pasteurisation	9	< 2 - 5.1	< 1 - 3.0	< 1	P **(1/9)**	< 2 - 3.3 **(3/9)**
16	End product: day of production	9	< 2 - 3.5	< 1 - 2.8	< 1 - 1.6	A	< 2 - 2.7

(a) TPAC: total psychrotrophic aerobic count; (b) ASC: aerobic spore count;(c) SRC: sulphite reducing Clostridia;

(d) LMO: *L. monocytogenes*; (e) A/P: Absent/Present in 25g or on 50cm^2

Table 2.9: Detailed results (minimum-maximum counts) for **type 3** REPFEDs based on compiled data of multiple companies. Counts are shown in log CFU/g for product samples (N° 1-7, 12, 15-16) and log CFU/25 cm^2 for environmental or contact surface samples (N° 8-11, 13-14). Results for *L. monocytogenes* relate to presence/absence testing. Numbers between brackets in bold indicate the number of samples that exceeded the legal criteria or microbial guide values. Numbers between brackets but not in bold indicates presence or above detection limit but compliance to the legal criteria and guide values.

N°	Sample description	n	Quality indicators			Pathogenic microorganisms	
		244	TPAC[a]	ASC[b]	SRC[c]	LMO[d]	*B. cereus*
1	Raw meat or fish	7	< 2 - 8.5 **(1/7)**	< 1	< 1 - 1.0	P[e] (2/7)	< 2
2	Raw vegetables or fruit	6	< 3 - 5.1	< 1 - 3.0	< 1	A[e]	< 2
3	Dry herbs and spices	8	-	< 2 - 5.9	< 1 - 3.3	-	< 2 - 4.0 **(2/8)**
4	Raw materials with safe harbour	6	< 1 - 3.0	< 1 - 1.0	< 1	A	< 2 - 2.0
5	Raw materials without safe harbour	12	< 2 - 7.4 **(2/12)**	< 1 - 2.3	< 1	A	< 2 - 2.0
6	Dry pasta or rice	8	4.4	< 1 - < 2	< 1 - 1.0	A	< 2 - 2.0
7	Powders or starches	7	-	< 1 - 3.0	< 1	-	< 2 - 3.1
8	Contact materials at raw material processing	12	0.5 - 5.1 **(3/12)**	< 0.5 - 0.5	-	A	< 1.5
9	Hands/gloves at raw material processing	4	< 0.5 - 6.0 **(2/4)**	< 0.5 - 0.5	-	A	< 1.5
10	Contact materials during preparation	42	< 0.5 - 5.4 **(25/42)**	< 0.5 - 2.6	-	P **(3/42)**	< 1.5
11	Hands/gloves during preparation	20	< 0.5 - 5.6 **(14/20)**	< 0.5 - 2.3	-	A	< 1.5
12	Prepared product prior to packaging	42	< 1 - 6.7 **(2/42)**	< 1 - 4.2	< 1 - 3.5	P **(1/42)**	< 2
13	Contact materials during packaging	34	< 0.5 - 4.1 **(5/34)**	<0.5 - 2.0	< 0.5	A	< 1.5
14	Hands/gloves during packaging	16	< 0.5 - 4.2 **(4/16)**	< 0.5 - 1.5	-	A	< 1.5
15	Packaged product prior to pasteurisation	2	< 2 - 2.0	< 1	< 1	A	< 2
16	End product: day of production	18	< 2 - 8.4 **(7/18)**	< 1 - 3/5	< 1	A	< 2

(a) TPAC: total psychrotrophic aerobic count; (b) ASC: aerobic spore count;(c) SRC: sulphite reducing Clostridia;

(d) LMO: *L. monocytogenes*; (e) A/P: Absent/Present in 25g or on 50cm^2

Table 2.10: Shelf life study results (minimum-maximum counts) **for type 1, 2 and 3 REPFEDs**. REPFEDs were prepared according to the instructions on the package. Counts are shown in log CFU/g. Numbers between brackets in bold indicate the number of samples that exceeded the legal criteria or microbial guide values.

REPFED-type	Sample description	n	Quality indicators			Pathogenic microorganisms	
		90	TPAC[a]	ASC[b]	SRC[c]	LMO[d]	*B. cereus*
1	End product: end of shelf life cold	18	< 2 - 6.3 **(1/18)**	< 1 - 3.8	< 1	A[e]	< 2
1	End product: end of shelf life: prepared	18	< 2 - 5.2	< 1 - 3.8	< 1	A	< 2
2	End product: end of shelf life cold	9	5.0 - 7.4 **(5/9)**	1.0 - 6.6	< 1 - 1.0	A	< 2 - 2.3
2	End product: end of shelf life: prepared	9	< 2 - 6.1 **(1/9)**	< 1 - 2.0	< 1	A	< 2
3	End product: end of shelf life cold	18	< 2 - 8.6 **(10/18)**	< 1 - 2.0	< 1	A	< 2
3	End product: end of shelf life: prepared	18	<2 - 6.6 **(2/18)**	< 1	< 1	A	< 2

(a) TPAC: total psychrotrophic aerobic count; (b) ASC: aerobic spore count;(c) SRC: sulphite reducing Clostridia;

(d) LMO: *L. monocytogenes*; (e) A/P: Absent/Present in 25g or on 50cm^2

Chapter 3

A Bayesian model for *Bacillus cereus* contamination in raw materials

Redrafted after:

Daelman, J., Membré, J. M., Uyttendaele, M. & Devlieghere, F. (2012). A bayesian model for *Bacillus cereus* contamination in raw materials used for cooked chilled food production. In *Belgian-Dutch Conference on Machine Learning (Benelearn)*, p. 62. Ghent (Belgium)

Summary

The first input required in the exposure assessment, is the prevalence or level of *B. cereus* in the raw materials of which the product is composed. The probability distributions fitted per ingredient will enable estimating *B. cereus* contamination and its variability in a crude product composition (e.g. 5% starch, 1% herbs, etc.). Microbiological analysis results (n=541) were collected from multiple REPFED companies. The dataset was divided in five groups: (i) starch components, (ii) dry herbs and spices, (iii) meat, fish and dairy products, (iv) fruits and vegetables and (v) ambient stable products. A hierarchical statistical model was set up and solved using a Bayesian Inference technique implemented in Winbugs, with a Monte Carlo Markov Chain algorithm. Model predictions were validated using actual data by comparing the observed and predicted percentage of samples exceeding the detection limit. The model resulted in five probability distributions for the contamination of *B. cereus* (log CFU/g), one for each group. Results were satisfactory even if the probability of exceeding the detection limit was slightly bigger than the actual 'positive' sample percentage. The model is used to estimate *B. cereus* concentration in raw materials in the QMEA in chapter 7.

3.1 Introduction

The quantitative microbial exposure assessment being developed in chapter 7 is based on the ICMSF risk-based framework (ICMSF, 2002) (Equation 3.1). In their equation, H_0 is the raw material contamination, ΣI the cumulative increase due to growth or recontamination, ΣR the cumulative decrease due to inactivation or removal and PO/FSO are the Performance and Food Safety Objective respectively.

$$H_0 + \Sigma I - \Sigma R \leq \text{PO or FSO} \tag{3.1}$$

From this equation it is clear that any comprehensive exposure assessment of REPFEDs should start with the raw material contamination (H_0). However, REPFEDs are complex products and they can contain as many as twenty ingredients. A single distribution for all these raw materials would mean that an exposure assessment for any product would start from the same *B. cereus* concentration, irrespective of product composition. This is unlikely, since it is known that certain types of raw materials are more frequently contaminated and/or contain higher concentrations than others. Examples are dried herbs, spices, pasta and rice (Powers *et al.*, 1976; Wijnands *et al.*, 2006).

Creating a set of probability distributions for groups of raw materials instead of one distribution

for all ingredients will allow tailoring the exposure assessment to any product recipe. It will also allow the assessment of changes in product composition or raw material contamination (e.g. decontamination of herbs). In this chapter a hierarchical Bayesian model is described, creating probability distributions of *B. cereus* in various raw material groups used in the production of REPFEDs.

3.2 Model development

3.2.1 Collection of microbiological data

Microbiological results for *B. cereus* in raw materials used in REPFED production (n=541) originated from five Belgian REPFED companies and comprised two parts: (i) historical microbiological analysis results (n=341) and (ii) new analysis of raw materials (n=200). The historical analysis results were from samples taken by the companies in 2009 in the framework of their respective food safety management systems. These samples were analysed by various accredited laboratories and reported back to the companies. No information about the microbiological methods used was available. The companies provided their complete *B. cereus* dataset for 2009. The new analyses of raw materials were performed as part of an assessment of the microbial safety of REPFEDs (Chapter 2). Samples were taken at the companies, stored cold (4°C) and analysed within 24 h according to ISO (2004) for *B. cereus* (plating on MYP agar (Oxoid) and incubating 24h at 30°C, confirmation on TSA with 5% Sheep Blood (BD, Erembodegem, BE)) (see Chapter 2).

3.2.2 Hurdles and assumptions

When the collected dataset was used to construct probability distributions of the *B. cereus* contamination, two hurdles were encountered. Firstly, there is a seemingly endless diversity in raw materials, which reduced the numbers of analysis per raw material to very low numbers (one or two at best). Secondly, the *B. cereus* concentration in 93.3% of the samples (505 of 541) was below the limit of detection (LoD) (Table 3.1). The LoD was between 5 and 100 CFU/g, depending on the method used by the different accredited laboratories.

Table 3.1: Number of analysis and number of censored data (< LoD) for the five raw material groups.

Category	Number of analysis	Number of analysis <LoD[a]
Starch component	64	60 (93.8%)
Dry herbs and spices	223	196 (87.9%)
Meat, fish and dairy products	137	133 (97.1%)
Fruit and vegetable products	89	88 (98.9%)
Ambient stable products	28	28 (100%)

(a) Limit of Detection

To cope with the large diversity in raw materials, the results were categorised in five raw material groups. The categorisation was based on the data gathered in chapter 2 combined with expert discussion and contained five groups:

1. Starch components (e.g. pasta, potatoes, rice, flour)
2. Dry herbs and spices (e.g. basil, garlic powder, pepper, nutmeg)
3. Meat, fish and dairy products (e.g. precooked sausages, minced salmon, grated cheese)
4. Fruit and vegetable products (e.g. mushrooms, frozen spinach, quorn, carrot cubes)
5. Ambient stable products (e.g. concentrated tomatoes, red wine, olive oil)

The problem of censored data was still present in the grouped data (Table 3.1). For example, the Ambient stable product group contained only negative samples (<1 *B. cereus* 25g). The different groups were linked together using an assumption about the origin of the variability in *B. cereus* contamination. Linking the different groups made it possible to estimate the probability distribution of the *B. cereus* concentration, even in groups with no positive samples. The basic assumption was that the variability in *B. cereus* contamination consisted of two parts: one part inherent to *B. cereus* and a second part inherent to the raw material group. Hence the variability difference between two groups will only depend on the difference in raw material groups and the variability within a group is constant, irrespective of the raw material group. This was translated into three distinct assumptions used for solving the hierarchical model (Figure 3.1):

1. The total variability in *B. cereus* contamination (N_i) is a combination of intra- and inter-group variability: $\log N_i \sim N(\mu_i, \sigma)$, μ_i is the average level of contamination for the group i (i = 1 to 5) and σ the standard deviation.

2. The intra- or within group variability (σ) is inherent to the variability in *B. cereus* contamination and is identical for all five groups. In the model, the precision ($P_\sigma = 1/\sigma^2$) is described by a Gamma distribution.

3. The inter- or between group variability (μ_i-μ_j (i ≠j)) is caused by the difference in product properties and is different for the five groups. Each μ_i is described by a Normal distribution: $\mu_u \sim N(M, S)$. In the model, M is described by a Normal distribution and the precision associated with S, $P_s = 1/S^2$, by a Gamma distribution.

Once set up, the hierarchical model was solved using a Bayesian inference technique chosen for its flexibility and because it allows the inclusion of censored data; no particular prior knowledge was incorporated in the estimation process.

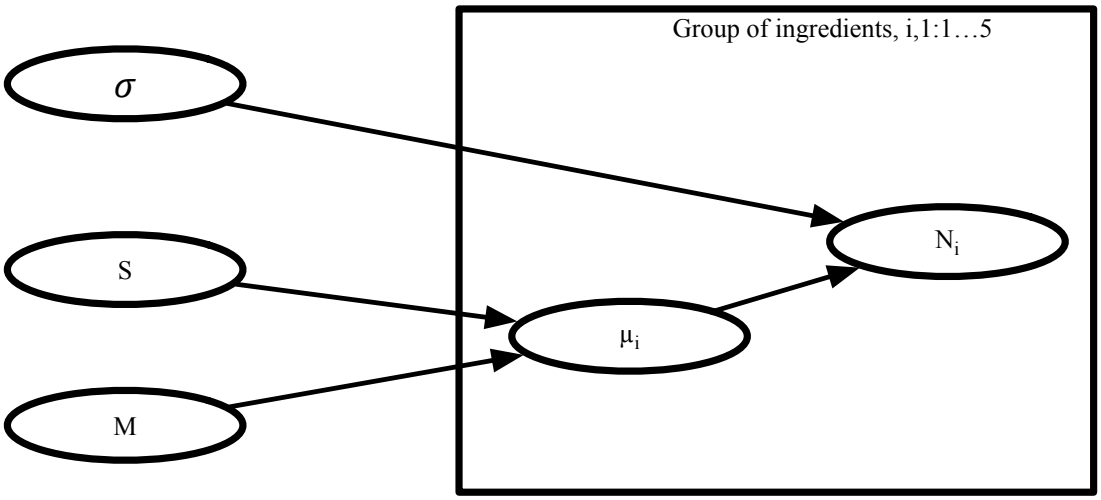


Figure 3.1: Directed acyclic graph illustrating the hierarchical model developed for analysing the raw material contamination dataset.

3.2.3 Estimation process and software

The Bayesian Markov Chain Monte Carlo procedure was run using Winbugs (version 1.4.3, Medical Research Council, UK). To check the convergence of the iteration process, visual analyses (history function and Gelman and Rubin diagnostic) of three independent chains were performed.

The model ran 100,000 iterations, of which the first 10,000 were eliminated as burn-in period. A visual analysis of three chains was performed to check the convergence of the iteration process and no problems were detected. The model outputs (posterior distributions) were visualised in Excel, using the add-in @Risk (Version 5.7.1, Palisade, USA) to build the histogram and calculate the percentage samples containing *B. cereus*.

3.2.4 Verification

The probability distributions were verified using various methods. Scatter plots were made for all combinations of parameters to check for correlations. The highest correlation values observed were 0.70 for μ_2 and μ_3 and 0.65 for μ_1 and μ_2. The probability distributions were compared to the actual data by plotting predicted percentage of positive samples (≥LOD for *B. cereus*) to the observed percentage of positive samples. Because the LoD in the observed data is variable, an average LoD (1.2 log CFU/g) was used to calculate the number of predicted positive samples.

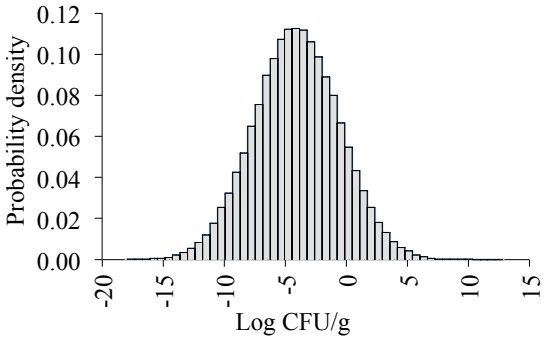
3.3 Results

3.3.1 Probability distributions

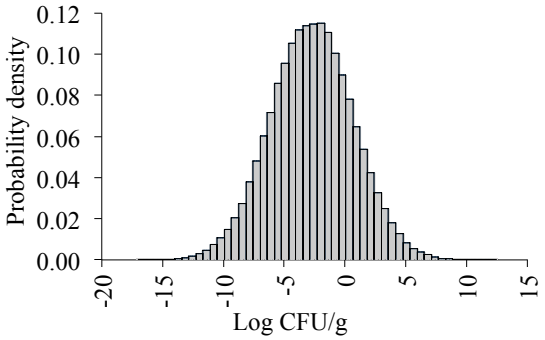
The hierarchical model resulted in five probability distributions, one for each raw material group. In each raw material group, the mean *B. cereus* contamination level, μ_i, was different (Table 3.2). This difference corresponds with the assumption about the intergroup (between) variability. The group 'dry herbs and spices' had the highest contamination level, the 'ambient stable products' the lowest. However, none of the groups were significantly different from the others as the credibility intervals systematically overlapped. The intra-group variability, σ, was estimated to 3.31 (95% CI: 2.50 - 4.43). With our assumption, this value remained constant, independent of the raw material group. Histograms of the *B. cereus* concentatrions in the five raw materials groups are presented in Figure 3.2.

Table 3.2: Probability distributions of each of the five raw material groups, values in log CFU/g.

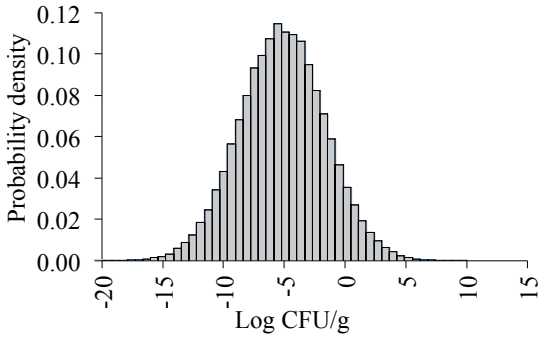
	$\log N_i \sim N(\mu_i,\sigma)$				
	$\mu_i \sim N(M,S)$			Credibility interval of μ_i	
Raw material group	M	S	σ	2.50%	97.50%
Starch components	-4.074	1.161	3.308	-6.67	-2.13
Dry herbs and spices	-2.727	0.766	3.308	-4.46	-1.47
Meat, fish and dairy products	-5.156	1.185	3.308	-7.85	-3.21
Fruit and vegetable products	-6.383	1.612	3.308	-10.08	-3.8
Ambient stable products	-7.139	3.15	3.308	-15.13	-3.39



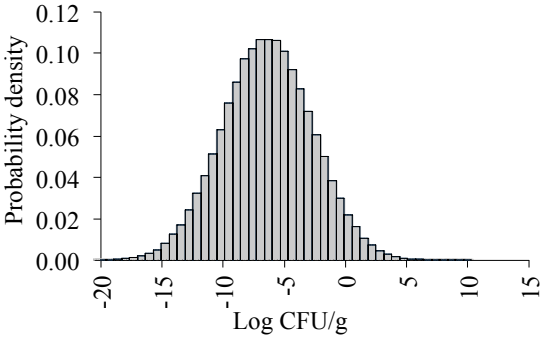
(a) Starch components



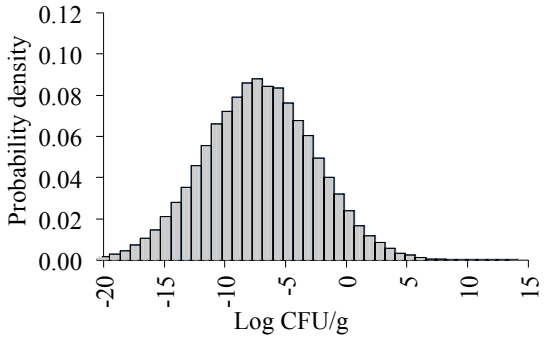
(b) Dry herbs and spices



(c) Meat, fish and dairy products



(d) Fruit and vegetable products



(e) Ambient stable products

Figure 3.2: Histograms for the *B. cereus* contamination in the five raw material groups:

3.3.2 Verification

Due to the significant portion of censored data it is not possible to directly compare the probability distributions with the observed data. Therefore, the predicted percentages of positive samples were compared to the observed percentages of positive samples (Figure 3.3). Although the percentage of predicted positive samples is slightly higher than the observed percentage, the results are satisfactory (R^2 0.993).

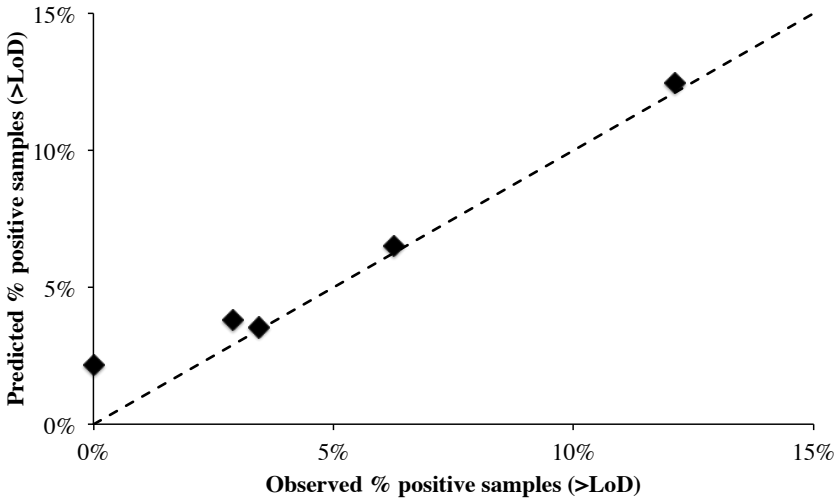


Figure 3.3: Predicted percentage of positive samples (≥ Limit of Detection (LoD)) for the five raw material groups as a function of the observed percentage of positive samples. Because the LoD was variable, an average value (1.2 log CFU/g) was used. Note that the percentages on the x-axis correspond to the complement of the values in table 3.1.

3.4 Discussion

Two hurdles were encountered when attempting to create distributions of the *B. cereus* contamination in raw materials. Despite the extensive set of data (n=541), a majority of the data was censored (i.e. corresponded to presence/absence information and not to actual numbers). The second hurdle was the large variety of raw materials. Then, with the objective of obtaining probability distributions ready to be implemented in the exposure assessment model (chapter 7), it was decided to develop a probabilistic model with two key assumptions: (i) data on some raw materials can be merged, resulting into only five groups of raw materials; (ii) the *B. cereus* contamination in log count can be described by a simple hierarchical model.

In this model, the five distributions were not considered as independent: the assumption of a constant intra-group variability (σ) was made; meaning that the dispersions around the average *B. cereus* contamination were considered as similar in the five raw material groups. In other words, the difference between the groups was considered to be only due to the mean level of contamination. This assumption enabled using the same template (hierarchical model with Normal and Gamma distributions) irrespective of the raw material group and then to standardise the statistical analysis. For example, the 'ambient stable products' group for which none of the 28 samples was positive and was kept in the analysis without any extra data handling. This model framework will make it possible to easily incorporate new raw material groups or new data if necessary.

To apply the same approach to another hazard or another food ingredient, the capacity to regroup the ingredients into homogeneous categories is key. Expert opinions are essential at this stage: without any prior information on the hazard contamination in the ingredients (expected prevalence and level, likely route of contamination...), it is difficult to categorise them.

Overall, the intragroup variability (σ) was estimated to 3.31, which is large and reflects the difficulty of dealing with binary data (positive/negative samples). The negative samples were not discarded, but included in the analysis as censored data (values lower than the LoD). Although the model provided satisfactory results with a correct prediction of the number of positive samples, the model outputs were not precise. For example, the *B. cereus* contamination of the 'ambient stable products' group was estimated to -7.1 (consistent with the fact that none of the samples was positive) but with a 95% credibility interval varying from -17.3 to 0.6. This large interval captures a part of natural variability, but also includes a large part of uncertainty due to the lack of data (only 28 data in this category, all of them corresponding to negative samples).

Despite this drawback, the Bayesian model provided interesting results: it ranked the five raw material groups in terms of *B. cereus* contamination level and confirmed that 'dry herbs and spices' are a relatively risky ingredient category. The comparison of intergroup variability (μ_i) is straightforward when a hierarchical model is set up, because the μ_i values are direct model outputs (Table 3.2).

The Bayesian inference is a flexible and easy-to-implement technique to analyse microbial contamination data. Indeed, binary data (number of positive/negative samples) were combined with figures (log counts in positive samples) to create continuous probability distributions, ready to be implemented in an exposure assessment model. Bayesian inference has been already used for constructing hierarchical models with applications in food microbiology or food safety (Buss-

chaert *et al.*, 2011; Crépet *et al.*, 2009; Membré & van Zuijlen, 2011) and more generally in QMRA (Delignette-Muller *et al.*, 2006). Bayesian techniques have been acknowledged as valuable methods to articulate probability distributions (e.g. contamination levels), uncertainty (e.g. due to censored data) and variability (e.g. due to biological materials) in a transparent manner.

Overall, results were satisfactory even if the probability of exceeding the detection - was slightly bigger than the actual positive sample percentage.

3.5 Conclusions

The bayesian model presented in this chapter provides an estimate of the *B. cereus* contamination in five raw material groups. Although the model-estimates are not always precise (e.g. large credibility intervals for ambient stable products), the probability distributions fitted per ingredient enable the estimation of the *B. cereus* contamination in a crude product composition (e.g. 5% starch, 1% herbs, etc.) and will be used in the exposure assessment model as H_0 input (Chapter 7).

Chapter 4

Growth/no growth models for heat-treated psychrotrophic *Bacillus cereus* spores under cold storage

Partially redrafted after:

Daelman, J., Vermeulen, A., Willemyns, T., Ongenaert, R., Jacxsens, L., Uyttendaele, M. & Devlieghere, F. (2013f). Growth no growth models for heat-treated psychrotrophic *Bacillus cereus* spores under cold storage. *International Journal of Food Microbiology*, 161(1):7–15

Summary

The microbial safety of REPFEDs is linked to spore-forming pathogens, more specifically *Clostridium botulinum* and *Bacillus cereus*. In this chapter two sets of growth/no growth (GNG) models are presented for heat treated spores of two *B. cereus* strains. The models incorporate both product parameters (a_w and pH) and process parameters (pasteurisation value at 90 °C (P_{90}) or heating temperature). The first set of models evaluate the effect of four different P_{90}-values (P_{90} = 0, 4, 7 or 10 min, all applied at 90°C) on the germination and subsequent growth of *B. cereus* spores under different conditions of pH and a_w at 10°C. These models show that a heat treatment not only increases the time-to-growth (TTG), but also significantly increases the minimal a_w and pH necessary for germination and subsequent growth. The second set of models for *B. cereus* spores compares the effect of three heat treatments, with the same P_{90}-value (10 min) but applied at different temperatures (85, 87 and 90°C), on the germination and subsequent growth at 10°C. The second model shows that lower heating temperatures (85 and 87°C) had less effect on the TTG, minimal a_w and minimal pH than a higher temperature (90°C). Finally, the first set of models was validated in broth using spores of seven psychrotrophic *B. cereus* strains, to evaluate the effect of strain variability on the model predictions.

4.1 Introduction

As mentioned in Chapter 1, to describe the situation of *B. cereus* in REPFEDs, predictive microbiological models must fulfil three criteria:

1. The model must be designed with spores, because vegetative cells will not survive the pasteurisation treatment (Byrne *et al.*, 2006)

2. The model must incorporate a heat treatment, because this will affect both lag time and growth (Gaillard *et al.*, 2005)

3. Lag time and growth should be measured under cold storage, because this is standard practice in the industry and it will affect the lag time and growth rate (Choma *et al.*, 2000a).

Because no suitable model was available in literature, this chapter presents two sets of growth/no growth (GNG) models for heat-treated spores of two psychrotrophic *B. cereus* strains under cold storage. In total four models were developed: two for each strain (i.e. one per model) and two for each type of model (i.e. one per strain). This first set of models (two models, one for each strain) uses a constant heat treatment temperature (90°C) and uses the P_{90}-value as variable. To

assess the validity of the P-value as a variable, this chapter presents a second set of models (two models, one for each strain). These models use a constant P_{90}-value (P_{90}=10) but use different heating temperatures (85, 87 and 90°C) and heating times. Finally the first set of models was validated in laboratory media, using spores of seven *B. cereus* strains: the two strains used for modelling and five other strains.

While the original intention was to use this model in the Quantitative Microbiological Exposure Assessment (QMEA) in chapter 7, the model output (growth probability in %) was to difficult to implement in this framework. The data gathered in this chapter was therefore expanded with additional data (at 8°Cand 30°C) and a new predictive model was developed (chapter 5).

4.2 Materials and methods

4.2.1 Preparation of growth media

For the development of the GNG model, sixteen different media were prepared based on Trypton Soy broth (TSB). These modified TSB media were heat sterilised (120°C for 15 min) and varied in pH (5.2 - 5.6 - 6.0 - 6.4) and a_w (0.973 - 0.980 - 0.987 - 0.995). These values were selected based on pH and a_w measurements of 45 different industrial REPFED products collected (Chapter 2). The water activity of the media was lowered by adding NaCl and verified prior to inoculation (a_w-kryometer AWK-20, NAGY messysteme GmbH, Gaüfelden, Germany). The pH of the medium was adapted using HCl and measured prior to inoculation (SevenEasy pH, Mettler-Toledo GmbH, Schwerzenbach, Switzerland).

4.2.2 Determination of the heat resistance

All experiments were performed using spores of two *B. cereus* strains (FF140 and FF355). Both strains were isolated from meal-components used in REPFED products, i.e. béchamel sauce and carrots respectively (Samapundo *et al.*, 2011c). To assess the difference in heat resistance between both strains, the D-values at 85, 90 and 95 °C and z-values of both strains were determined. The values for strain FF140 were previously determined by Samapundo *et al.* (2011c). The values for strain FF355 were determined using the same procedure. In short: the spores were inoculated in 100 ml of preheated TSB (pH 7.0) to reach a level of $10^{6\text{-}7}$ spores per ml. Flasks were heated at the desired temperature in a warm water bath and 1 ml samples were taken at regular intervals and cooled quickly. Surviving spores were determined by plate counting on Trypton Soy Agar (TSA). This procedure was performed in duplicate. D and z-values were determined

by linear regression in SPSS Statistics 20.

4.2.3 Inoculum preparation and inoculation

Strains were taken from a stock culture at -75°C, inoculated in 9 ml of TSB and incubated for 24 h at 30°C. To standardise the inoculum, a second subculture was taken and incubated for 24 h at 30°C. Strain purity was checked by plating on TSA and *B. cereus* was confirmed by plating on Mannitol egg Yolk Polymyxine agar (MYP).

Spores were generated using a method based on that of Coroller *et al.* (2001): 100 μL of a secondary subculture was inoculated on strengthened nutrient agar (sNA, 28 g/l nutrient agar, 0.04 g/l $MgCl_2$, 0.10 g/l $CaCl_2$). Five plates were prepared per strain and incubated for 5 days at 30°C. Spores were collected by bringing 4 ml of sterile salt solution (8.5 g/l NaCl) on the surface and gently rubbing the surface of the agar with a sterile spatula. The suspension was collected from all five plates and combined in a 50 ml Falcon tube. This process was repeated to remove remaining spores. The spores were then washed by centrifugation (10.000 g, 15 min) and the supernatant was removed and resuspended in 10 ml of sterile NaCl solution. This process was repeated three times and after the third washing the pellet was resuspended in 10 ml of a 50% (v/v) ethanol solution and stored for 1 h at 2°C. After storage the spores were washed another three times by centrifugation and after each cycle the pellet was resuspended in 10 ml of sterile distilled water. Presence of spores was confirmed by plating twice on TSA and incubating 24 h at 30°C: once without heat treatment, once after the sample was heated 10 minutes at 80°C to eliminate vegetative cells. The final suspension contained $10^{7\text{-}9}$ spores/ml and was stored at 2°C for a maximum of 4 weeks to minimise the effect of spore age (Collado *et al.*, 2003b).

The experiments were performed in heat resistant microplates (Microplate 96/F-PP, Eppendorf, Hamburg, Germany). Before inoculation the wells of the microplates were filled with 180 μl of modified TSB (i.e. TSB with modified pH and a_w). Each plate contained four media with identical a_w and different pH. To avoid changes in the modified TSB during inoculation, due to dilution with unmodified TSB, the spores were diluted in the modified TSB prior to inoculation (i.e. a specific inoculum was made for each pH a_w combination). After dilution, 20 μl of specific inoculum was added to the wells to obtain a final inoculum concentration of $10^{5\text{-}6}$ spores/ml. The different heat treatments caused an approximate reduction in spore concentration of 1 log, so the final inoculum after heat treatment and before the start of cold storage was $10^{4\text{-}5}$ spores/ml. Microplates that were not heat-treated were inoculated at $10^{4\text{-}5}$ spores/ml. The inoculum density was verified by plating on TSA. The microplates were then sealed using a self-adhesive transparent film (Viewseal nonpiercable, Greiner bio-one).

4.2.4 Heat treatment and storage

The microplates were heated using a Thermostat Plus heating block (Eppendorf) with microplate adaptor and cover. To prevent heat loss, the space between the plate and the cover was filled with a piece of expanded polystyrene (EPS) that was cut to size. The time-temperature profiles that were used, were tested using uninoculated microplates. To mimic the conditions of the actual experiment as close as possible and to prevent evaporation, a septum was inserted between the EPS and the film (Viewseal). The thermocouple was passed through the EPS, the septum and the film. The temperature was recorded using a data logger (Testo 177-T4, Testo, Ternat, Belgium).

Pasteurisation values at 90°C (P_{90}) were calculated using equation 4.1 with a reference temperature (T_{ref}) of 90°C and a z-value of 9.55°C (mean of both strains, Table 4.1). To minimise the variation in the temperature profile due to difference in the heating-up process, the plates were placed on a preheated thermoblock. After a set time at the given temperature, the thermoblock actively cooled the plates until 10°C. The temperature registration and calculation of the P_{90}–value started when the cold plate was put on the heating block (at 10°C) and was stopped after heat treatment, when the temperature had dropped back to 10°C.

$$P_{90} = \int_0^t 10^{\left(\frac{T-T_{ref}}{z}\right)} dt \tag{4.1}$$

Seven heat treatments were tested in this study: one without heat treatment (N° 1: no heating or P_{90}=0), three treatments with the same maximal heating temperature (90°C), designed to yield a P_{90} of 4, 7 and 10 minutes (N° 2-4) and three treatments with the same P_{90}-value but a different maximal temperature of 85°C, 87°C and 90°C (N° 5-7). The fourth and the seventh heat treatment are identical. Schematic time-temperature-profiles of the seven different heat treatments are shown in Figure 4.1. Once the plates had cooled down, the film was replaced with a regular microplate lid in order to remove the condensation formed during the heating process. The plates were subsequently stored for 65 days at 10°C under aerobic conditions. Although common practice in the industry is storage 4-8°C, a temperature of 10°C was used, to be able to perform all experiments within a reasonable time frame. Although the vegetative cells of both strains can grow aerobically at 7°C (Samapundo *et al.*, 2011b), preliminary experiments showed that the strains were unable to grow anaerobically at 8°C. Under aerobic conditions growth was slower and less frequent (more no growth) at 8°C than at 10°C. To facilitate modelling experiments were therefore incubated at 10°C.

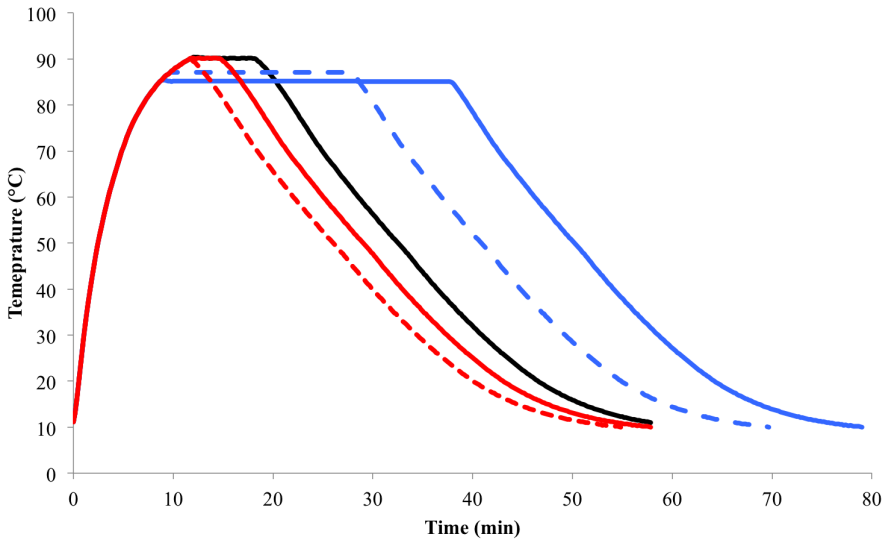


Figure 4.1: Schematic representations of the seven time-temperature profiles used. Set 1 (heating temperature = 90°C): P_{90}=10 (——); P_{90}=7 (—); P_{90}=4 (- - -). Set 2 (P_{90}=10 min): 90°C (——); 87°C(- - -); 85°C(——).

4.2.5 Data generation

The optical density (OD) of the media was measured at 600nm using a Versamax microplate reader (Molecular devices, Sunnyvale, CA, USA) and the data were processed using the SOFTmaxPRO software package (Molecular devices). All combinations were performed in 8 replicates. The wells were measured three times a week for a period of 65 days, resulting in at least 20 data points for each replicate of a medium. Prior to each measurement the microplates were shaken for 2 minutes at 600 rpm (MTS 2/4 digital microtitershaker, IKA, Staufen, Germany). In order to define growth, the OD of a well was compared to the OD_{zero} of this combination (of a_w, pH and P_{90}). The OD_{zero} is the average of all 8 wells at time zero. A well (of a certain combination) was considered to show growth, if the difference between the OD of a well and the OD_{zero} of the combination was larger than three times the standard deviation of the OD_{zero}. Data were processed using Excel (Microsoft, Redmond, Virginia, USA), a process that was automated using a custom written Excel macro application. All wells were visually verified for possible false positive results (e.g. air bubbles) and all OD-curves were checked for anomalies. For each condition the probability of growth was calculated as the number of wells showing growth over the number of wells tested. E.g. if 2 out of 8 replicates showed growth, the probability of growth was 25%.

4.2.6 Development of the growth/no growth models

The growth/no growth data were used to develop four models; two sets of models for each of the two *B. cereus* strains (FF140 and FF355). For both types of models an ordinary logistic regression model was used to describe the data. The first type model (Eq. 4.2) incorporated a_w, pH, pasteurisation-value at 90°C (P_{90}) and storage time. The second type of model (Eq. 4.3) incorporated a_w, pH, storage time and heat treatment temperature.

$$\begin{aligned} logit(p) = {} & b_0 + b_1 \cdot a_w + b_2 \cdot pH + b_3 \cdot P_{90} + b_4 \cdot time + b_5 \cdot a_w^2 + b_6 \cdot pH^2 \\ & + b_7 \cdot P_{90}^2 + b_8 \cdot time^2 + b_9 \cdot a_w \cdot pH + b_{10} \cdot a_w \cdot P_{90} + b_{11} \cdot a_w \cdot time \\ & + b_{12} \cdot pH \cdot P_{90} + b_{13} \cdot pH \cdot time + b_{14} \cdot P_{90} \cdot time \end{aligned} \tag{4.2}$$

$$\begin{aligned} logit(p) = {} & b_0 + b_1 \cdot a_w + b_2 \cdot pH + b_3 \cdot Htemp + b_4 \cdot time + b_5 \cdot a_w^2 + b_6 \cdot pH^2 \\ & + b_7 \cdot Htemp^2 + b_8 \cdot time^2 + b_9 \cdot a_w \cdot pH + b_{10} \cdot a_w \cdot Htemp + b_{11} \cdot a_w \cdot time \\ & + b_{12} \cdot pH \cdot Htemp + b_{13} \cdot pH \cdot time + b_{14} \cdot Htemp \cdot time \end{aligned} \tag{4.3}$$

In these equations, $logit(p) = ln(p/(1-p))$ with p the probability of growth (p ∈ [0,1]), pH and a_w are the pH and the water activity of the medium respectively; P_{90} is the pasteurisation-value at 90 °C calculated using equation 4.1; *time* is the storage time at 10 °C in days, *Htemp* is the heat treatment temperature (85, 87 or 90°C) and b_i (i=0, ..., 14) are the parameters being estimated. The models were fitted in SPSS 19.0 (SPSS Inc., Chicago, IL, USA) using linear logistic regression according to the procedure described in Vermeulen *et al.* (2007a). In short, this procedure means that the main effects (a_w, pH, time and P_{90} or Heat treatment temperature) are forced to stay in the model irrespective of their significance (p-value). The quadratic and interaction terms were selected by backwards-stepwise regression based on the likelihood criterion (p=0.001). The resulting growth/no growth interfaces were plotted in Matlab¨7.13 (The Mathworks, Inc., Natick, MA, USA).

Goodness-of-fit statistics considered were: (i) -2 ln((*L*)with *L* the likelihood in its optimum, (ii) Akaike's Information Criterion (AIC = -2 ln(*L*) + 2*k*, with *k* the number of parameters in the model) and (iii) Schwartz Criterion (SC = -2 ln(*L*)+ $k \cdot \ln(n)$ with *n* the number of observations). The predictive power was measured using the c-value: the concordance index or the area under the ROC-curve (Receiver Operating Characteristic-curve). The ROC curve is a plot of the sensitivity (proportion of success that is correctly predicted) against 1-specificity (proportion of failures that is correctly predicted). The further the ROC lies above the reference line, the more

accurate the model is. The area under the curve can be estimated using the concordance index. It estimates the probability that the predictions and the outcomes are concordant. A value $c = 0.5$ means that the predictions are no better than random guessing and the higher the value of c, the better the prediction (Agresti, 2002).

4.2.7 Model validation in broth with other *B. cereus* strains

To evaluate the importance of strain variability on the applicability of the model, the first set of growth/no growth models was validated using spores of seven *B. cereus* strains: the two strains used for modelling (FF140 and FF355) and five other strains (FF137; FF143, FF206, FF306 and LFMFP307). All strains were isolated from different REPFEDs or meal components used in REPFED production. Ten combinations (Table 4.4) of a_w, pH and P_{90}-value were selected and tested in 20 replicates for each strain. The combinations were chosen based on their location in relation to the GNG boundary, i.e. the majority of the combinations were in the no growth zone, or on the GNG boundary. Procedures for generation of spores, medium preparation, inoculation, heat treatment, storage and detection were identical to those used in the initial data gathering.

The validity of the models was assessed using four criteria after 30 and 60 days. (i) The percentage correct predicted: % of replicates with growth predicted as growth, and no growth predicted as no growth. (ii) The percentage fail-safe: % of replicates predicted as growth, but showing no growth. (iii) The percentage fail-dangerous: % of replicates predicted as no growth but showing growth. (iv) The concordance index (c-value or area under the Receiver Operating Characteristic (ROC)).

To account for the fact that all conditions were close to the growth/no growth boundary a secondary validation of the model was performed. In this validation a predicted growth percentage $\geq$0.1% was considered as complete growth.

4.3 Results

4.3.1 Heat resistance of the *B. cereus* spores used for model development

There is a significant difference in heat resistance between spores of the two strains (Table 4.1). Spores of strain FF140 are the most heat resistant of the two and have a $D_{90°C}$-value of 90.9 min, while spores of strain FF355 are about 5 times more heat sensitive with a $D_{90°C}$-value of 17.9 min. Both strains have similar z-values of 9.6 °C and 9.5 °C respectively.

Table 4.1: D- and z-values for *B. cereus* strains FF355 and FF140 with 95% confidence interval

Strain	$D_{85°C}$ (min)	$D_{90°C}$ (min)	$D_{95°C}$ (min)	z-value (°C)
FF140[a]	293.3 [250.0, 500.0]	90.9 [76.9, 111.1]	26.6 [24.4, 28.6]	9.6 [8.1, 11.6]
FF355	58.8 [47.6, 76.9]	17.9 [15.9, 20.8]	5.2 [4.0, 7.6]	9.5 [8.4, 10.9]

[a]Samapundo *et al.* (2011c)

4.3.2 Models with P_{90} as variable and constant heating temperature

A four dimensional (a_w, pH, P_{90}, storage time) GNG model (Eq. 4.2) was fitted for each of the strains. All heat treatments for these models were performed at 90°C. The estimated parameters with their standard deviation and goodness-of-fit statistics for both models are given in Table 4.2.

Results show that at 10°C, spores of both strains are not able to germinate and grow at the lowest pH (5.2) but are able to grow at the lowest water activity (0.973) or after the most intense heat treatment tested (P_{90} = 10 min). To quantify the effect of a stress factor on the growth probability in function of time, time-to-growth (TTG) was defined as the time when the growth probability is equal to 10%. An increase in P_{90}-value increases the TTG, but also causes an increase in the minimal a_w and pH for growth (Figures 4.2 and 4.3). However, the effect of a heat treatment on these variables is not straightforward. When the P_{90}-value increases, the marginal effect on a_w, pH and TTG decreases (Figure 4.2).

From the cross sections at constant pH (Figure 4.2) it is clear that an increase in P_{90}-value has a larger effect on strain FF355 than on strain FF140, which can be explained by the difference in $D_{90°C}$-value between the two strains (Table 4.1). In addition, for strain FF355 the effect of the heat treatment is more dependent on the water activity than for strain FF140 (Figure 4.3). For strain FF140 there is little difference in the effect of a heat treatment at a_w 0.980 or a_w 0.955 (Figure 4.3a), while the same a_w difference gives a remarkable effect on the growth probability of strain FF355 (Figure 4.3b).

The effect of the different factors (a_w, pH and P_{90}) is larger at the beginning of the storage. After approximately 30 days of storage, the GNG interphase does not evolve any more as a function of time. For example, Figure 4.3a shows the minimal pH for strain FF140 in function of the time for the different heat treatments. From these cross sections it is clear that the minimal pH for germination and growth decreases as a function of time for the first 30 days, but does not change anymore afterwards.

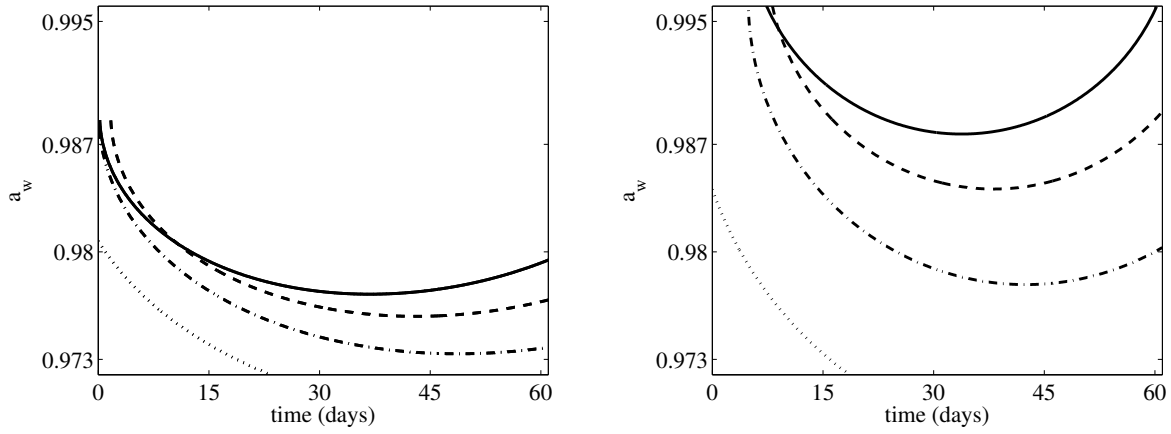
Table 4.2: Parameter estimates with their standard errors and performance statistics for the first model (variables: a_w, pH, storage time and P_{90} value and constant heat treatment temperature)

Variable[a] **/Performance statistic**	**Parameter est. ± st. error Strain FF 140**	**Parameter est. ± st. error Strain FF 355**
Intercept	$-3.72 \cdot 10^2 \pm 1.26 \cdot 10^2$	$-1.91 \cdot 10^2 \pm 1.17 \cdot 10^2$
a_w	$7.43 \cdot 10^2 \pm 2.55 \cdot 10^2$	$3.77 \cdot 10^2 \pm 2.39 \cdot 10^2$
P_{90}	$-4.24 \cdot 10 \pm 3.80 \cdot 10^{-1}$	$-4.41 \cdot 10 \pm 2.87 \cdot 10$
pH	$1.68 \cdot 10^2 \pm 8.91 \cdot 10$	$1.20 \cdot 10^2 \pm 7.89 \cdot 10$
$time$	$-4.53 \cdot 10 \pm 4.70 \cdot 10^{-1}$	$-2.20 \cdot 10^{-1} \pm 8.00 \cdot 10^{-2}$
a_w^2	$-3.76 \cdot 10^2 \pm 1.29 \cdot 10^2$	$-1.90 \cdot 10^2 \pm 1.21 \cdot 10^2$
P_{90}^2	$6.50 \cdot 10^{-2} \pm 4.60 \cdot 10^{-3}$	$7.10 \cdot 10^{-2} \pm 6.00 \cdot 10^{-3}$
pH^2	$-1.37 \cdot 10 \pm 7.40 \cdot 10^{-1}$	$-9.72 \cdot 10 \pm 6.50 \cdot 10^{-1}$
$time^2$	$-3.90 \cdot 10^{-3} \pm 1.60 \cdot 10^{-4}$	$-4.60 \cdot 10^{-3} \pm 2.00 \cdot 10^{-4}$
a_w x $time$	$3.60 \cdot 10 \pm 4.30 \cdot 10^{-1}$	N.S.
P_{90} x a_w	N.S.[b]	$3.57 \cdot 10 \pm 2.69 \cdot 10$
P_{90} x pH	$5.50 \cdot 10^{-1} \pm 6.00 \cdot 10^{-2}$	$1.25 \cdot 10 \pm 8.30 \cdot 10^{-2}$
P_{90} x $time$	$-2.00 \cdot 10^{-2} \pm 9.20 \cdot 10^{-4}$	$-1.30 \cdot 10^{-2} \pm 1.10 \cdot 10^{-3}$
pH x $time$	$2.50 \cdot 10^{-1} \pm 1.40 \cdot 10^{-2}$	$1.10 \cdot 10^{-1} \pm 1.30 \cdot 10^{-2}$
Number of observations	1985	1748
-2 ln(*L*)	3340.074	3050.012
AIC	3366.074	3076.012
SC	3438.7	3147.07
Hosmer-Lemeshow	8.155	8.423
	p-value = 0.418	p-value = 0.393
% correct predictions	95.4	95.4
c-value	0.993	0.99

[a] Variables not listed were not significant (p = 0.01) for both strains

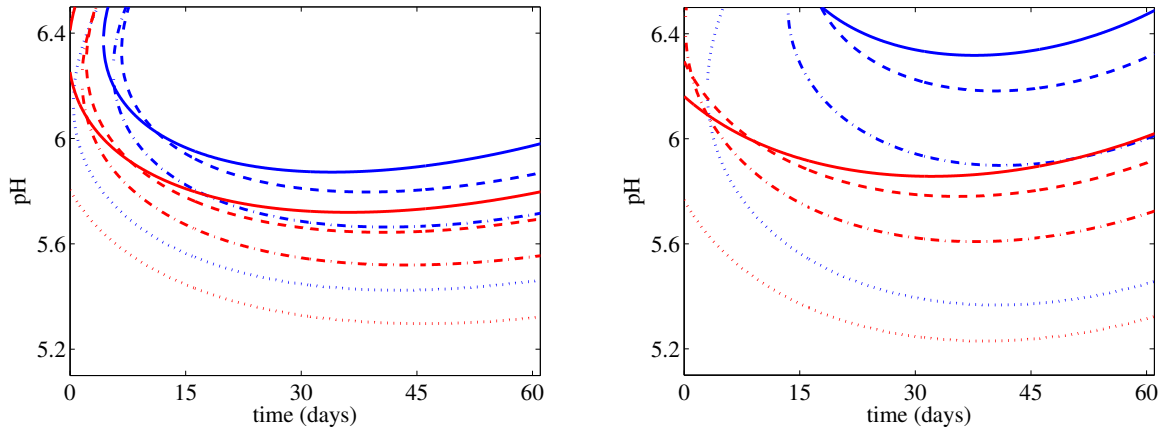
[b] N.S.: Not significant (p = 0.01)

The model shows that it is ineffective to use only product formulation (a_w, pH) or only pasteurisation to prevent germination and growth of *B. cereus* spores. To prevent germination and growth without heat treatment, the pH must be as low as 5.2 On the other hand, even the most intense heat treatment tested (P_{90}=10) is not able to prevent germination and growth at maximal pH and a_w (6.4 and 0.995 respectively). A combination of stringent conditions for all factors is the most efficient way to reduce the growth probability. For strain FF140 applying a P_{90} of 10 minutes at optimal pH (6.4) and a_w (0.995) will not increase the TTG compared to not applying a heat treatment (Figure 4.4a). However, when the pH is lowered to 6.0 and the water activity to 0.980 a heat treatment with a P_{90} of 4 min can extend the TTG with approximately seven days (Figure 4.4b), which is ± 1/3 of the average shelf life of the REPFED. Similar results were obtained for strain FF355.



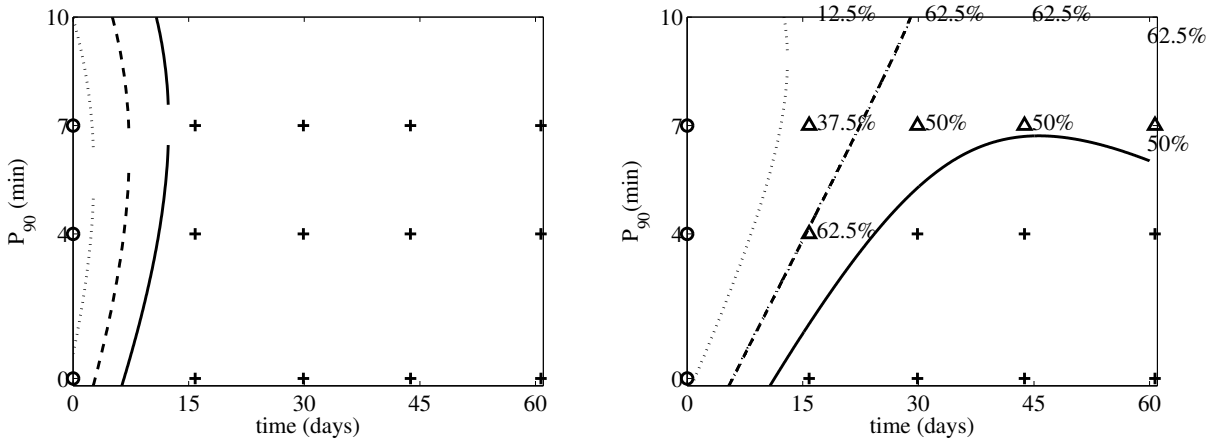
(a) strain FF140 **(b)** strain FF355

Figure 4.2: Growth/no growth boundaries at pH 6.0 for 4 different heat treatments, (a) strain FF140, (b) strain FF 355. Lines represent the ordinary logistic regression model predictions (p=0.10) the growth zone is situated above and to the right of the lines: P_{90} = 0 min (· · ·); P_{90} = 4 min (- · -); P_{90} = 7 min (- - -); P_{90} = 10 min (—).



(a) strain FF140 **(b)** strain FF355

Figure 4.3: Growth/no growth boundaries at constant a_w for 4 different heat treatments (P_{90} = 0 min (· · ·); P_{90} = 4 min (-·-·); P_{90} = 7 min (- - -); P_{90} = 10 min (—)) and two different water activities (blue = a_w 0.980, red = a_w 0.995) for (a) strain FF140 and (b) strain FF355. Lines represent the ordinary logistic regression model predictions (p=0.10), the growth zone is situated above and to the right of the lines.



(a) a_w 0.995 - pH 6.4 **(b)** a_w 0.980 - pH 6.0

Figure 4.4: Growth/no growth boundaries for strain FF140 at (a) a_w 0.995 and pH 6.4 and (b) a_w 0.980 and pH 6.0. Lines represent the ordinary logistic regression model predictions p=0.9 (—), p=0.5 (- - -), p=0.1 (· · ·). Points are actual data: (+) p = 1, (o) p=0 and (△) $p \in]0,1[$ with the measured percentage of growth indicated.

4.3.3 Models with heating temperature as variable and P_{90}-value constant

The first model (section 4.3.2) used P_{90}-values applied at a maximal temperature of 90°C. To confirm whether the results of the first model remain valid for a P_{90} applied at lower maximal temperatures (87 and 85°C) a new 4 dimensional GNG model (Eq. 4.3) was fitted for each of the strains (variables: a_w, pH, heat treatment temperature (*Htemp*) and storage time). Heat treatments had the same P_{90}-value (10 min), but were performed at three different temperatures (85, 87 and 90°C). The estimated parameters with their standard deviations and goodness-of-fit statistics for both models are given in Table 4.3.

The models for both strains show a large effect of the heating temperature on the growth-probability. The effect of pasteurisation (P_{90}=10) on the minimal pH for germination and subsequent growth decreases with decreasing heat treatment temperature (Figure 4.5). A comparison between model 1 and 2 for strain FF355 after 60 days, shows that pasteurising at 85 °C for a given time (approx. 38 min - Figure 4.1) to achieve a P_{90} of 10 min results in the same minimal pH (5.6) for growth (p=0.10) as pasteurising at 90 °C with a P_{90}-value of 4 minutes (Figure 4.3b). The effect of heating temperature is less pronounced for strain FF140 than for strain FF355.

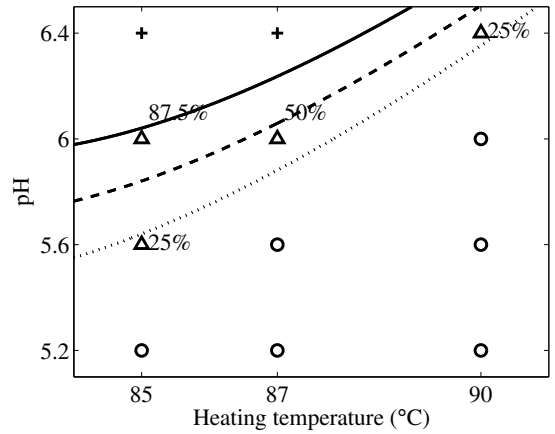


Figure 4.5: Growth/no growth boundaries after 60 days at a_w 0.995, for strain FF355. Lines represent the ordinary logistic regression model predictions p=0.9 (—), p=0.5 (- - -), p=0.1 (· · ·). Points are actual data: (+) p = 1, (o) p=0 and (△) $p \in$]0,1[with the measured percentage of growth indicated.

The heat treatment temperature also affects the growth probability (Figure 4.6). When strain FF140 is heat-treated at 90 °C (P_{90}=10, a_w 0.955, pH 5.6), none of the eight replicates showed growth after 60 days and the model predicts a ±20% probability of growth (from 30 until 60

days). When the same heat treatment is applied at 87°C, the model and the data show 50% growth after 15 days. At 85 °C the probability of growth is even larger and all replicates showed growth after less than 15 days.

Table 4.3: Parameter estimates with their standard errors and performance statistics for the second model (variables a_w, pH, storage time and heat treatment temperature and constant P_{90}-value)

Variable[a] **/Performance statistic**	**Parameter est. ± st. error Strain FF 140**	**Parameter est. ± st. error Strain FF 355**
Intercept	$4.87 \cdot 10^2 \pm 3.03 \cdot 10^2$	$-5.14 \cdot 10^2 \pm 1.83 \cdot 10^2$
a_w	$1.05 \cdot 10^2 \pm 5.89 \cdot 10$	$1.12 \cdot 10^2 \pm 3.89 \cdot 10^2$
pH	$-7.26 \cdot 10 \pm 4.40 \cdot 10^{-1}$	$-5.33 \cdot 10 \pm 9.74 \cdot 10$
time	$4.85 \cdot 10 \pm 3.67 \cdot 10$	$4.40 \cdot 10^{-1} \pm 1.40 \cdot 10^{-1}$
Htemp[b]	$-9.06 \cdot 10^2 \pm 7.11 \cdot 10$	$-8.09 \cdot 10 \pm 5.65 \cdot 10$
a_w^2	$-8.38 \cdot 10 \pm 4.80 \cdot 10^{-1}$	$-6.14 \cdot 10^2 \pm 2.12 \cdot 10^2$
pH^2	$-2.50 \cdot 10^{-3} \pm 1.40 \cdot 10^{-4}$	N.S.
$time^2$	$-8.80 \cdot 10^{-2} \pm 1.50 \cdot 10^{-2}$	$-3.60 \cdot 10^{-3} \pm 1.90 \cdot 10^{-4}$
$Htemp^2$	$7.92 \cdot 10 \pm 4.40 \cdot 10^{-1}$	$-1.90 \cdot 10^{-1} \pm 1.90 \cdot 10^{-2}$
a_w *x time*	$-3.40 \cdot 10 \pm 2.79 \cdot 10$	N.S.
Htemp x a_w	$1.70 \cdot 10^{-1} \pm 1.10 \cdot 10^{-2}$	$1.11 \cdot 10^2 \pm 5.97 \cdot 10$
pH x time	$-1.50 \cdot 10^{-2} \pm 1.30 \cdot 10^{-3}$	$7.90 \cdot 10^{-2} \pm 1.20 \cdot 10^{-2}$
Htemp x time	$4.87 \cdot 10^2 \pm 3.03 \cdot 10^2$	$7.00 \cdot 10^{-1} \pm 1.10 \cdot 10^{-1}$
Htemp x pH	N.S.[c]	$-6.90 \cdot 10^{-3} \pm 1.70 \cdot 10^{-3}$
Number of observations	1373	1307
-2 ln(L)	4026.248	2769.499
AIC	4052.248	2793.499
SC	4120.17	2855.60488
Hosmer-Lemeshow	1426	37.166
	p-value = 0	p-value = 0
% correct predictions	93.4	94.1
c-value	0.98	0.983

[a] Variables not listed were not significant (p = 0.01) for both strains

[b] Heat treatment temperature, [c] N.S.: Not significant (p = 0.01)

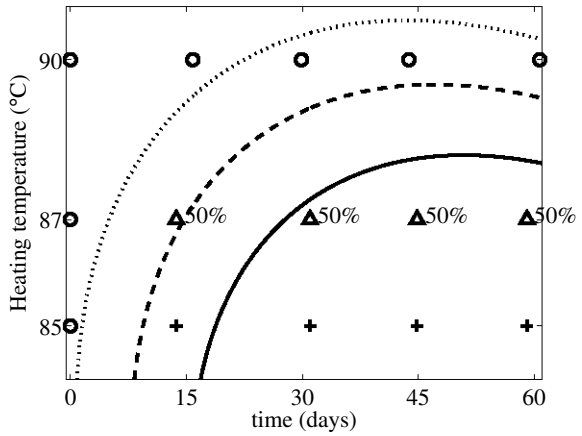


Figure 4.6: Growth/no growth boundaries for strain FF140 for a_w 0.995 and pH 5.6. Lines represent the ordinary logistic regression model predictions p=0.9 (—), p=0.5 (- - -), p=0.1 ($\cdot\cdot\cdot$). Points are actual data: (+) p = 1, (o) p=0 and ($\triangle$) $p \in$]0,1[with the measured percentage of growth indicated.

4.3.4 Model validation in broth with other *B. cereus* strains

The first set of growth/no growth models was validated using spores of seven other *B. cereus* strains. Ten combinations (Table 4.4) of a_w, pH and P_{90}-value were selected and tested in 20 replicates for each strain. The results of the validation after 60 days are displayed in Table 4, results of the validation after 30 days are not shown since they are similar to the results of the validation after 60 days. The percentage of replicates that is correctly predicted is acceptable (between 66% and 79%). However, results show that both models had a rather high fail dangerous rate (FD) (i.e. the number of replicates showing growth that were predicted as no growth). The model for strain FF140 (Model 1) had a %FD of 17% after 30 days and 18% after 60 days. For FF355 (Model 2) results were worse: 26% after 30 days and 34% after 60 days. This means that after 60 days, the model for FF355 incorrectly predicted no growth for wells that showed growth in one third of the replicates.

It should be noted that the % correct predicted has to be interpreted with due attention as the percentage of growth can only take discrete values dependent on the number of replicates (Vermeulen *et al.*, 2007b), which influences the accuracy of the model validation. The model can predict any value from 0% to 100%, but the number of replicates in the validation (n=20) is limited and the smallest difference is 5% (1/20). As a practical example, if the model predicts a 87.5% growth probability the best result validation can give is 85% (17/20) or 90% (18/20). In addition, most combinations of stress factors (a_w, pH, P_{90}) in the validation were chosen close to,

or even within the GNG interphase. Because this region of the experimental range is more subject to variability, this can strongly bias the criteria proposed for model evaluation (see section 4.2.7). If for example the combinations for the validation had been chosen at the extremes of the experimental range, for example very low a_w and pH or high P_{90} where growth is very unlikely, the results of the validation would have been better.

From an industrial point of view, the probability of growth is less important than the location of the combination with respect to the GNG boundary. For use in a risk or exposure assessment, a GNG model is difficult to apply as a function of time, because it predicts a probability of growth rather than a simple "yes" or "no". This implies that a choice has to be made about what growth probability is tolerable. To test if the model is more accurate when forcing it to give a "yes or no" answer, a 0.1% threshold is proposed. If the probability of growth is less than 0.1%, the model is said to predict no growth (0%). If the probability of growth is equal or larger than 0.1%, the model is said to predict growth (100%). Using this threshold the model predictions are converted to a binary form. This adjustment also makes the model more realistic for industrial application. As an example, for combination 5 (a_w 0.995, pH 5.6 and P_{90}=4) the model for strain FF140 gives a growth-probability of 29% after 30 days and 35% after 60 days (Table 4.4). Using the threshold value of 0.1% these values are converted to 100% growth-probability. For strain FF140 this means that the %FD (for this combination, model and strain) decreases from 61% (35%) at 30 days (60 days) to 0%. When the model predictions are converted according to this rule the %FD (Table 4.4 "adapted"-values) drops drastically for the models of both strains.

Detailed analysis showed that the %FD could not be attributed to a specific strain (e.g. more heat resistant). This would be the case if one of the strains was more resistant than all the others. The %FD could also not be attributed to a specific combination (i.e. a_w, pH, P_{90}). If this were the case it could be caused by an error in the media making or in the heat treatment. The %FD is spread over all strains and over all combinations that show growth.

Table 4.4: Overview of the media-heat treatment combinations after 60 days for seven different *B. cereus* strains: Data (%) are the percentage growth during data-collection for modelling; Predictions (%) are the growth probabilities predicted by the GNG models; Strain-data (%) are the observed growth probability for each strain during validation. The lower part of the Table lists the criteria for model validation.

	Conditions			**Data (%)**		**Predictions (%)**		**Strain - data (%)**						
N°	a_w	pH	P_{90}	M1[a]	M2[b]	M1	M2	**FF 140**	**FF 355**	**FF 137**	**FF 143**	**FF 206**	**FF 306**	**LFMFP 307**
1	0.973	5.2	0	0	0	0	0	0	0	0	0	0	10	0
2	0.987	6	0	100	100	100	100	100	100	100	100	100	95	100
3	0.973	5.6	4	0	0	0	0	0	0	10	0	0	5	15
4	0.98	6.4	4	100	100	100	90.3	100	100	100	100	100	100	100
5	0.995	5.6	4	25	0	35	0.9	90	80	45	55	0	25	15
6	0.973	6.4	7	100	0	42.8	0.1	100	85	50	40	100	30	45
7	0.98	5.6	7	0	0	0	0	75	70	0	20	85	10	10
8	0.987	6	7	100	37.5	99.6	5.2	100	100	40	70	100	75	100
9	0.973	6	10	0	0	0	0	75	40	20	40	90	30	25
10	0.987	5.6	10	0	0	0	0	100	80	30	20	100	25	5

	Total (all strains)		**FF 140**		**FF 355**		**FF 137**		**FF 143**		**FF 206**		**FF 306**		**LFMFP 307**	
Criteria	**M1**	**M2**	**M1**	**M2**	**M1**	**M2**	**M1**	**M2**	**M1**	**M2**	**M1**	**M2**	**M1**	**M2**	**M1**	**M2**
c-value	-	-	0.813	0.889	0.934	0.944	1	0.944	1	0.778	0.813	0.889	1	0.889	0.938	0.778
% Correct	78.7	66.2	63.7	45.7	72.2	54.2	86.3	80.2	86.8	75.2	63.2	52	86.8	78.2	92.2	78.2
% fail safe	2.9	0.1	0	0	0	0	6	0	3.2	0	3.5	0.1	5.2	0.5	2	0
% fail dangerous	18.4	33.7	36.3	54.4	27.8	45.9	7.72	19.9	10	24.9	33.3	47.9	8	21.4	5.8	21.9
% correct - adapted	75	75	74	74	77.5	77.5	77.5	77.5	78.5	78.5	62.5	62.5	74.5	74.5	80.5	80.5
% fail safe - adapted	10.9	10.9	1	1	3.5	3.5	16.5	16.5	13.5	13.5	10	10	17.5	17.5	14	14
% fail dangerous - adapted	14.1	14.1	25	25	19	19	6	6	8	8	27.5	27.5	8	8	5.5	5.5

[a] GNG model for *B. cereus* strain FF140 with variables a_w, pH and P_{90}.

[b] GNG model for *B. cereus* strain FF355 with variables a_w, pH and P_{90}.

4.4 Discussion

Psychrotrophic *B. cereus* is an important pathogen in REPFEDs because of its ability to survive the heat treatment and grow at low temperatures (Carlin *et al.*, 2000a). The heat treatment commonly used to guarantee the safety of REPFEDs (P_{90}=10) was originally designed for inactivation of psychrotrophic *C. botulinum*. Due to the difference in heat resistance between these two microorganisms it is unsure that this treatment will also inactivate spores of psychrotrophic *B. cereus* strains (Gibbs, 2002).

As mentioned in the materials section, the P_{90}=10 heat treatment has a limited lethal effect (1 log) on the spores of *B. cereus*, but it does increase the time-to-growth. However, pasteurisation itself is insufficient to prevent germination and growth of *B. cereus* spores during the shelf life periods generally given to these products: 10-45 days. The same is true for lowering water activity or pH. Within the a_w-range (0.977 - 0.997) or pH-range (5.1 - 6.5) of REPFEDs (Chapter 1 & 2), it is nearly impossible to prevent growth without pasteurising. However, it must be noted that the psychrotrophic *B. cereus* strains used in the present study are very heat resistant (Samapundo *et al.*, 2011c). The first set of models shows that there is no simple relationship between the P_{90}-value and the growth probability. The marginal effect of a heat treatment decreases, as the heat treatment is longer. For example the same difference in P-value (3 min) does not yield the same change in minimal pH. This is illustrated in Figure 4.3b where the vertical distance between the lines decreases as the heat treatment increases. The models also show that lowering the pH of the product has a significant effect when this is used in combination with a heat treatment.

REPFED producers are looking for ways to reduce the heat treatment, to improve product quality, reduce vitamin losses or allow higher throughputs. There are two ways to reduce heat treatment: (i) reducing heat treatment time or (ii) reducing heat treatment temperature.

The second model (variables: a_w, pH and heating temperature) illustrates that the effect of a P_{90}-value on the growth probability also depends on the heating temperature. This means that using lower temperatures for longer times to achieve the same P-value should be done with caution. While these treatments may have the same lethal effect (P_{90}), their effect during storage may vary significantly. According to the models, high temperature short time treatments have more effect on germination and growth of *B. cereus* spores during storage at low temperature than longer heat treatments at lower temperature. A possible cause for this increased growth after heat-treating at lower temperature may be an increased activation of the spores at lower temperatures compared to more inactivation of the spores at higher temperatures (Collado *et al.*, 2003b; Turnbull *et al.*, 2007). This effect was less pronounced for the more heat resistant strain FF140 ($D_{90°C}$ = 90.9

min) than for the heat sensitive strain FF355 ($D_{90°C}$ = 17.9 min). Because this strain is generally less sensitive to heat treatments the difference in effect of different heat treatments is smaller than for the more heat sensitive strain (Figure 4.2).

Since most REPFEDs have an extended shelf life, it is useful to have a model that informs about the microbial stability as a function of time, as the shelf life period itself is an important factor in guaranteeing food safety. As demonstrated in the validation, the results of these GNG models should be interpreted carefully. If the model predicts a 1% probability of growth this means that 1 in every 100 products will allow germination and growth of *B. cereus* spores and this is unacceptable for manufacturers. A 0.1% cut-off was selected, to be more realistic for the industry, for they would prefer the lowest possible growth probability. On the other hand a logistic regression is asymptotic, a value lower than 0.1% will result in unrealistic conditions of pH, a_w and P_{90}. To account for the probabilistic nature of a GNG model, it is important to take into account the distance between a certain combination of stress factors (P_{90}, pH and a_w) and the growth/no growth boundary. The further a certain combination (e.g. pH, a_w and P_{90}) is from the GNG boundary, the smaller the chance of growth, and thus the larger the safety margin.

As listed in the introduction, a model to describe *B. cereus* in the REPFED production should fulfil three criteria: use spores instead of vegetative cells, apply a heat treatment to simulate pasteurisation and incubate at low temperature to simulate the cold chain. The model presented in this chapter is the first model for *B. cereus* to fulfil all three criteria. The importance of these criteria can be clearly illustrated by comparing the results in this chapter with an existing model (e.g. www.combase.cc). For a product with a_w 0.980 and pH 6 stored at 10 °C that is not heat-treated (P_{90}=0), the difference between both models is limited. This may indicate that at moderate a_w and pH and without heat treatment, the difference between vegetative cells and spores is limited; Combase predicts a lag phase of 97 hours or 4 days while the GNG models predict growth after 2-3 days (10%). However, for the same product but pasteurised (P_{90}=10) the Combase prediction will not change (4 days) while the GNG models will predict growth after 10 days (strain FF140) or even no growth (strain FF355). Combase is a suitable model for non-heat-treated *B. cereus* but is fail-safe and will given unrealistic short shelf life estimates when used for heat-treated *B. cereus* spores.

Finally it is important for the industry that the GNG models are fail-safe because the *B. cereus* spores were incubated at 10°C, a temperature which corresponds to mild temperature abuse. Lower storage temperatures will most likely result in longer TTG values and smaller growth-regions. In addition to the effect incubation temperature the effect of inoculum size must be taken into account. In this study an inoculum concentration of $10^{4\text{-}5}$ spores/ml was used, which

is considerably higher than the *B. cereus* concentrations generally reported in food products (Carlin *et al.*, 2000b; Del Torre *et al.*, 2001). Multiple authors have suggested that increased spore concentration increase the probability of germination (Abee *et al.*, 2011; Caipo *et al.*, 2002; Peck & Stringer, 2005). Given the following reasons, it is possible that the risk of *B. cereus* in REPFEDs has been systematically over-estimated: (i) most existing models under-estimate the lag phase or over estimate the possibilities for growth of heat-treated *B. cereus* spores at low temperatures; (ii) the germination and growth of heat-treated *B. cereus* spores is limited, even under conditions of mild temperature abuse (10 instead of 7°C), (iii) that *B. cereus* is usually present in low concentrations and (iv) that most REPFEDs are MAP or vacuum packed and recent research as well as or own data (unpublished) suggest that *B. cereus* has difficulties to grow at cold temperatures under anaerobic conditions (de Sarrau *et al.*, 2012). In addition many commercial products contain organic acids to inhibit microbial growth. These acids will also affect growth and heat resistance of *B. cereus* (Leguerinel & Mafart, 2001; Mols *et al.*, 2010).To correctly estimate the risk, additional research simulating the actual conditions in REPFEDs is needed (e.g. low inoculum, MAP, heat treatment, low temperature storage, etc.).

4.5 Conclusions

The growth/no growth models presented in this chapter are a first attempt to model the behaviour of heat-treated *B. cereus* spores under cold storage. The models show that heat-treatment has limited impact under optimal conditions and is most effective when combined with other stress-factors, especially pH. In addition the models illustrate that heating at high temperatures for a short time is more effect than lower temperatures for longer times. The validation showed that while strain variability has a considerable impact on the accuracy of the model predictions, the % correct is sufficiently high to localise the GNG boundary. The main drawback of GNG models is that a tolerable growth probability has to be selected for application in an exposure assessment. While the original intention was to use the GNG model in the QMEA in chapter 7, the model output (growth probability) proved too difficult to implement in this framework. The data gathered in this chapter was therefore expanded with additional data (at 8°C and 30°C) and a new predictive model was developed (chapter 5).

Chapter 5

Development of a time-to-detect growth model for heat-treated *Bacillus cereus* spores

Partially redrafted after:

Daelman, J., Aditya, S., Vermeulen, A., Uyttendaele, M., Devlieghere, F. & Membré, J. M. (2013a). Development of a time-to-detect growth model for heat-treated *Bacillus cereus* spores. *International Journal of Food Microbiology*, 165(3):231–240

Summary

In this chapter, a predictive model is developed, describing the time-to-detect growth of psychrotrophic *Bacillus cereus* spores submitted to various combinations of pH, a_w, heat treatment and storage temperature. The dataset of 434 combinations (of pH, a_w, heat treatment, storage temperature and *B. cereus* strain), which was originally collected to build a growth/no-growth model for two *Bacillus cereus* strains (chapter 4), was re-interpreted as time-to-detect growth values. The model had a Gamma multiplicative structure and was solved by Bayesian inference with informative prior distributions. To be implemented in a decision tool, for instance to calculate the process and formulation conditions required to achieve a given detection time, each Gamma term had some constraints: they had to be monotonous, continuous and algebraically simple mathematical functions (i.e. having analytical solution). Overall, the cumulative effect of various stressful conditions (pasteurisation process, low temperature, low pH) made it possible to extend the time-to-detect growth up to 60 days or more, whereas the heat-treatment on its own did not have a similar effect.

5.1 Introduction

The extent to which *B. cereus* may pose a safety risk within a REPFED will depend on many factors including: spore prevalence and concentration in raw materials, heat treatment, heat resistance of spores, product formulation and supply-chain storage temperatures (Membré & Lambert, 2008). In particular, the effect of the heat treatment and product formulation on the subsequent lag time of surviving spores can have a significant impact on the safe shelf-life (Barker *et al.*, 2005; Faille *et al.*, 1997; Laurent *et al.*, 1999; Martinez *et al.*, 2007).

To our best knowledge, there is no off-the-shelf predictive model describing the combination of thermal processing and product formulation on *B. cereus* spore lag time. Likewise, there is not much data publicly available showing the effect of both heat-treatment and formulation conditions on *B. cereus* spores. When searching in ComBase (Baranyi & Tamplin, 2004) for log count growth curves (selected criteria: *B. cereus*, culture medium, temperature below or equal 20°C), the lag time values were relatively short even under stressful conditions, for instance, at temperature 7°C and pH 5.5, lag times were less than one week (record B130_59 and B130_60, data from Campden and Chorleywood Food Research Association).

The objective of this study was to develop a mathematical model quantitatively assessing the effect of the factors related to the production process (heat treatment), the product formulation (pH

and a_w) and the environment (chilled storage temperature, or alternatively ambient temperature during product preparation) on the lag time of heat-treated *B. cereus* spores. However the spore lag time, i.e. the sum of time required to have spore germination and outgrowth, was not measured directly. Instead, the growth/no-growth dataset (at 10°C) previously generated in chapter 4 was expanded with new data (at 8°C and 30°C) and reanalysed to extract the time before detecting growth. The limit of detection of the turbidimetry method used to generate the data was 1.3 10^6 CFU/ml, and consequently the spore inoculum was deliberately high (10^4-10^5) to achieve the detection limit quickly. Obviously, strictly speaking, the time before detecting growth (hereafter referred to as 'detection time') is always longer than the spore lag time. However, with a high inoculum, the outgrowth time is short in comparison to the lag time and the detection time is relatively close to the spore lag time. A total of 434 data was analysed. To enable a further use of the model in determining the various conditions of formulation and processing that guarantee detection times longer than a desired value (e.g. 30 days or 50 days), the mathematical model chosen had three constraints:

1. To be continuous in the model domain of use.
2. To be based on monotonous functions for each factor.
3. To remain algebraically simple.

These constraints enable to directly determine a single heat-treatment process suitable to deliver a given detection time for a specific formulation (pH and a_w), or vice-versa (one formulation for a specific process). This application was considered as highly valuable for further process and formulation optimisation, either carried out with the time-to-detect growth model run on its own, or incorporated in a more comprehensive farm-to-fork risk assessment model.

5.2 Materials and Methods

5.2.1 The dataset

The experimental protocol is described in chapter 4. The experiments were performed using 8 wells from a microtitre plate reader for each condition. When growth occurred in at least 2 wells at time t, the detection time was defined at t-1 (longest time where no growth was observed in 7 wells), this was an observed detection time. On the other hand, when no visible growth was observed in any of the 8 wells, the detection time was considered to be longer than the time when the experiment was stopped (60 days or more), and analysed as right-censored data.

The detection time of two strains of *B. cereus* isolated from REPFEDs, strain 1 (FF140 isolated from béchamel sauce) and strain 2 (FF355 isolated from carrots) was studied as a function of the factors heat treatment (time and temperature), pH, a_w and storage temperature. Spores of strain 1 have a $D_{90°C}$-value of 90.9 min, while spores of strain 2 have a $D_{90°C}$-value of 17.9 min. Both strains have similar z-values of 9.6 °C and 9.5°C, for strains 1 and 2, respectively (Chapter 4, Table 4.1). In Table 5.1, the experimental conditions are provided, for each factor and each strain. From the 434 data points collected on *B. cereus* spores, 223 corresponded to observed detection time values and 211 to censored data. Among the 223 observed values, detection times varied between 0.2 and 56.6 days. A set of 26 data presented detection times lower than or equal to 1 day while the factors heat treatment, pH, a_w and storage temperature were not altogether at their optimal values (Table 5.2). For example, detection times of 1 day were obtained at storage temperature of 10°C and pH 5.6 when the heat-treatment condition was mild (85°C or 87°C for 1 min).

The initial inoculum of the two strains before heat-treatment was chosen so that after thermal inactivation, a heat-treated spore concentration of 10^4-10^5 CFU/ml was obtained for strain 1 and strain 2. Since the heat treatment was applied after spore inoculation, the spores were in the same medium, and the same pH and a_w conditions, during the whole experiment (from initial inoculation to 60 days or more).

Table 5.1: Dataset used to build the model: heat-treatment (HT) temperature and time, *B. cereus* strains, status (censored data or lag times), a_w, pH and storage temperature conditions.

HT temp.[a]	Strain	Status and range of heat treatment time [min-max]	product and processing factors [min-max]
212 data collected at 85°C	89 data on strain 1	33 data (lag time value) for HT time of [1 -38] min	aw [0.973 -0.995] pH [5.2 -6.4] Storage temp.[b] [8.0 -10.0]
		56 censored data (> 60 d) for HT time of [1 -38] min	aw [0.973 -0.995] pH [5.2 -6.0] Storage temp. [8.0-10.0]
	123 data on strain 2	50 data (lag time value) for HT time of [1 -38] min	a_w [0.973-0.995] pH [5.2-6.4] Storage temp. 10.0°C
		73 censored data (> 60 d) for HT time of [1 -38] min	a_w [0.973-0.995] pH [5.2 -6.0] Storage temp. [8.0 -10.0]
119 data collected at 87°C	59 data on strain 1	34 data (lag time value) for HT time of [1 -38] min	aw [0.973 -0.995] pH [5.2 -6.4] Storage temp. [8-30]
		25 censored data (> 60 d) for HT time of [1 -38] min	a_w [0.973-0.995] pH [5.2-5.6] Storage temp. [8-10]
	60 data on strain 2	29 data (lag time value) for HT time of [1-38] min	a_w [0.973-0.995] pH [5.6 -6.4] Storage temp. [10-30]
		31 censored data (> 60 d) for HT time of [1-38] min	a_w [0.973-0.995] pH [5.2-6.4] Storage temp. [8-10]
103 data collected at 90°C	47 data on strain 1	38 data (lag time value) for HT time of [1-38] min	a_w [0.973-0.995] pH [5.2-6.4] Storage temp. [10-30]
		9 censored data (> 60 d) for HT time of 38 min	a_w [0.973 -0.995] pH [5.2 -6.0] Storage temp. [10-30]
	56 data on strain 2	39 data (lag time value) for HT time of [1 -38] min	a_w [0.973-0.995] pH [5.2-6.4] Storage temp. [10-30]
		17 censored data (> 60 d) for HT time of [10-38] min	a_w [0.973-0.995] pH [5.2 -6.4] Storage temp. [10-30]

[a] Heat treatment temperature (°C), [b] Storage temperature (°C)

5.2.2 The time-to-detect growth model applied to heat-treated spores

The model used to predict the detection time (time from inoculation to first growth observation) of heat-injured spores of *B. cereus* has been adapted from the set of equations developed for predicting non-proteolytic *C. botulinum* spore lag time (Membré, 2012). The general framework follows the Gamma concept originally suggested for microbial growth rate (Wijtzes *et al.*, 1998; Zwietering, 2002). The detection time was described by a general equation, with multiplicative terms (Eq. 5.1). The natural logarithm transformation of the response, i.e. of the detection time value, was chosen to stabilise the variance.

$$ln\,(lag + 1) = a_s \cdot \prod_{i=1}^{k} \gamma_{is} - 1 + \varepsilon \tag{5.1}$$

In Eq. 5.1, "lag" corresponded to the time-to-detect growth, explained for a large part by the spore lag phase but included as well vegetative cell growth up to a detectable level. There were four γ_i terms corresponding to the four factors (k=4): storage temperature (StT), pH, a_w and heat treatment (HT, itself split into heat-treatment time, HT_t and heat-treatment temperature, HT_T). The index "s" corresponded to the strain (s=1 or 2). The residual error term, ε, was assumed to be Normally distributed: $\varepsilon \sim \mathcal{N}(0, \sigma_\varepsilon^2)$. The left hand side of Eq. 5.1 was written as $ln(lag + 1)$ to enable the logarithm transformation even with detection time values reported as zero.

There are two main modifications in comparison with a Gamma structure. The first one lies in the addition of a term "-1" in Eq. 5.2. Indeed, with this "-1" term, the right hand side of Eq. 5.1 could be negative, particularly when the factors StT, pH, a_w, HT_t and HT_T were equal, or close to their optimal values. Likewise, this additional term "-1" enabled to get a detection time nil (in Eq. 5.1, $ln(lag + 1) = 0$ is equivalent to $lag = 0$) when the factors were not at their optimal values and meant that the coefficient (a_s) did not correspond to the minimal detection time observed. However, this mathematical parameterisation was chosen to include in the model all the data reporting very short detection times (detection time equal to 1 day or less) at sub-optimal conditions (Table 5.2). Overall, the parameterisation chosen here meant that the Gamma model *sensu stricto* over-estimated the detection time and had to be moderated (Eq. 5.2):

$$lag + 1 = exp\left(a_s \cdot \prod_{i=1}^{k} \gamma_{is}\right) / exp\,(1) \tag{5.2}$$

Moreover, the model parameterisation (Eq. 5.1) means that the model is applicable only to detection time data obtained in days. It would have been different with data expressed, for

Table 5.2: Experimental conditions leading to a detection time lower than or equal to 1 day.

Strain	HT_T [a] (°C)	HT_t [b] (min)	StT [c] (°C)	pH	a_w	Observed Detection time (days)
1	85	1	10	5.6	0.995	1
				6	0.987	1
					0.995	1
				6.4	0.98	1
					0.987	1
					0.995	1
	87	1	10	5.6	0.995	1
				6	0.987	1
					0.995	1
				6.4	0.987	1
					0.995	1
		38	10	6.4	0.995	1
	90	38	22	5.6	0.987	0.98
					0.995	0.86
	87	38	30	5.6	0.987	0.47
				6	0.987	0.23
				6.4	0.987	0.18
	90	38	30	5.6	0.987	0.43
					0.995	0.38
				6	0.973	0.69
				6.4	0.973	0.5
2	87	38	30	5.6	0.987	0.53
				6	0.987	0.3
				6.4	0.987	0.27
	90	38	30	5.6	0.987	0.72
					0.995	0.74

[a] Heat treatment temperature, [b] Heat treatment time, [c] Storage temperature

instance, in hours. The second difference with the lag model previously developed for bacterial spores (Membré, 2012) is a conditional effect of heat-treatment to the pH: the heat-treatment extended the detection time if, and only if, the pH was lower than its optimal (Eq. 5.3). This assumption was made based on the conclusion in chapter 4 with the same two strains: it was observed that the heat-treatment effect was accentuated at lower pH conditions.

$$\begin{cases} \prod_i^k \gamma_{is} = f_{1s}(StT) \cdot f_{2s}(pH) \cdot f_{3s}(a_w) \cdot f_{4s}(HT) & \text{if pH} \leq \text{pH}_{opt} \\ \prod_i^k \gamma_{is} = f_{1s}(StT) \cdot f_{2s}(pH) \cdot f_{3s}(a_w) & \text{if pH} > \text{pH}_{opt} \end{cases} \tag{5.3}$$

Concerning the two strains, the model was built with the following assumptions: the residual error, ε, was the same whatever the strain. This error encompassed experimental error, model adjustment error and natural variability in spore response. On the other hand, the strain response to a combination of stress (low storage temperature, low pH, low a_w) was considered as dependent on the strain. This assumption was also based on the results of chapter 4, where we highlighted a difference between the two strains in both minimal a_w and minimal pH. The effect of the four factors was expressed mathematically by a monotonous function as indicated below (Eqs. 5.4 to 5.10).

$$f_{1s}(StT) = \begin{cases} \to \infty & \text{for } StT \leq T_{min,s} \\ \left(\frac{T_{opt}-T_{min,s}}{StT-T_{min,s}}\right)^{0.5} & \text{for } T_{min,s} < StT \leq T_{opt} \\ 1 & \text{for } StT > T_{opt} \end{cases} \tag{5.4}$$

$$f_{2s}(pH) = \begin{cases} \to \infty & \text{for } pH \leq pH_{min,s} \\ \left(\frac{pH_{opt}-pH_{min,s}}{pH-pH_{min,s}}\right) & \text{for } pH_{min,s} < pH \leq pH_{opt} \\ 1 & \text{for } pH > pH_{opt} \end{cases} \tag{5.5}$$

The effect of a_w on the detection time was assessed firstly through a general pattern, including a specific effect of a_w on strain 1 ($f_{31}(a_w)$) and strain 2 ($f_{32}(a_w)$) as summarised in Eq. 5.6.

$$f_{3s}(a_w) = \begin{cases} \to \infty & \text{for } a_w \leq a_{w,min,s} \\ \left(\frac{a_{w,opt}-a_{w,min,s}}{a_w-a_{w,min,s}}\right) & \text{for } a_{w,min,s} < a_w \leq a_{w,opt} \\ 1 & \text{for } a_w > a_{w,opt} \end{cases} \tag{5.6}$$

However the effect of a_w on strain 2 was not established, i.e. there was no change in the deviance information criterion, nor in the residual model error when a model with or without $f_{32}(a_w)$ term. Consequently, it was decided to simplify the model as follows (Eq. 5.7):

$$f_{3s}(a_w) = \begin{cases} \begin{cases} \to \infty & \text{for } a_w \le a_{w,min,1} \\ \left(\frac{a_{w,opt}-a_{w,min,1}}{a_w-a_{w,min,1}}\right) & \text{for } a_{w,min,1} < a_w \le a_{w,opt} \\ 1 & \text{for } a_w > a_{w,opt} \end{cases} & \text{for strain 1} \\ 1 & \text{for strain 2} \end{cases} \tag{5.7}$$

The heat-treatment effect was split into heat-treatment time (HT_t) and heat-treatment temperature, (HT_T) (Eq. 5.8-5.10). This effect was considered as independent of the strains.

$$f_{4s}(HT) = f_{4s}(HT_T) \cdot f_{4s}(HT_t) \tag{5.8}$$

$$f_{4s}(HT_T) = \begin{cases} \to \infty & \text{for } HT_T \ge HT_{max} \\ \left(\frac{HT_{max}-HT_{opt}}{HT_{max}-HT_T}\right)^{0.5} & \text{for } HT_{opt} \le HT_T < HT_{max} \\ 1 & \text{for } HT_T < HT_{opt} \end{cases} \tag{5.9}$$

$$f_{4s}(Ht_t) = = \begin{cases} \to \infty & \text{for } HT_T \ge HT_{max} \\ t^p & \text{for } HT_{opt} \le HT_T < HT_{max} \\ 1 & \text{for } HT_T < HT_{opt} \end{cases} \tag{5.10}$$

In Eq. 5.10, the coefficient p is a shape coefficient. When the model was developed, several values for this coefficient were tested successively (deviance information criterion), the final value was set to 0.1.

The coefficients HT_{opt}, T_{opt} and $a_{w,\text{opt}}$, although model parameters, were not estimated but fixed to the following values: HT_{opt} = 85°C, T_{opt} = 37°C, $a_{w,\text{opt}}$= 0.999. This was done to avoid an over-parameterisation of the model equation system. During the first stage of modelling, an attempt was made estimate all the parameters (e.g. also HT_{opt}, $a_{w,\text{opt}}$ and T_{opt}). However, this resulted in high correlation values (most likely because of the synergistic effects between pH, heating and a_w). The number of parameters being estimated was then reduced until the correlation values were acceptable. Only the parameters that made sense from a biological point of

view (e.g. $pH_{\min}$) were estimated, because these can be verified based on literature or experimental data. On the other hand, the parameter pH_{opt} utilised in the model as a rule (below pH_{opt}, heat-treatment effect considered, above pH_{opt}, no heat-treatment effect) was estimated. The coefficients $a_1, a_2, T_{\min,1}, T_{\min,2}, pH_{\min,1}, pH_{\min,2}, a_{w,\min,1}, HT_{\max}$ and the residual standard deviation σ_ε were the other parameters estimated by statistical inference.

5.2.3 Bayesian inference

To solve the model equation system containing ten estimated parameters a Bayesian approach was chosen for two reasons. Firstly, among the 434 data, 211 were right-censored, meaning that the data recording was stopped before any microbial growth was detected. It appeared natural to express the information provided by the censored data in terms of probability (Eq. 5.11) and consequently to use an inference method dealing explicitly with probability distribution functions:

$$P_r\{\text{Detection time} \geq \text{time when the experiment was stopped}\} \rightarrow 1 \tag{5.11}$$

Secondly, despite a relatively large amount of data, some additional information was necessary to solve the model equation system. Bayesian techniques enable the incorporation of previous knowledge on the model parameters into the estimation process, through the settings of prior probability distributions.

The model parameters $T_{min,1}$, $T_{min,2}$, $pH_{min,1}$, $pH_{min,2}$, pH_{opt}, $a_{w,min,1}$, HT_{max} were assumed to follow a Normal distribution, $\mathcal{N}$(mean, variance). Normal distributions for the model parameters are often chosen in Bayesian statistics. The statistical reason for this choice lies in the following mathematical property: with a Normal likelihood and a Normal prior, the posterior distribution is also a Normal distribution (Marin & Robert, 2007). This property is an algebraic convenience; otherwise a difficult numerical integration may be necessary during the model inferring step.

To set the mean of the prior distribution, information provided by either the EFSA (2005a) or by Experts was used. The variance of the prior was set as follows. Firstly, it was assumed that the temperature varied in a range of a few units, pH in a range of a few units and tenths, a_w in a range of a few hundredths and thousandths. Secondly, the prior distributions, although informative, were kept rather wide since the information on *B. cereus* collected in the literature (e.g. $T_{\min}$) corresponded to values obtained in absence of heat-treatment stress. Thirdly, as the values given were rough estimates, to simplify the model implementation, only powers of ten were chosen (10^1, 10^{-1},10^{-2}).

$$T_{min,1} \sim \mathcal{N}(4, 10) \tag{5.12}$$

$$T_{min,2} \sim \mathcal{N}(4, 10) \tag{5.13}$$

$$pH_{min,1} \sim \mathcal{N}(4.5, 0.1) \tag{5.14}$$

$$pH_{min,2} \sim \mathcal{N}(4.5, 0.1) \tag{5.15}$$

$$pH_{opt} \sim \mathcal{N}(6.5, 0.1) \tag{5.16}$$

$$a_{w,min,1} \sim \mathcal{N}(0.92, 0.01) \tag{5.17}$$

$$HT_{max} \sim \mathcal{N}(120, 10) \tag{5.18}$$

To have the coefficient a_1 and a_2 positive, they were taken into account in the statistical inference process after the following mathematical transformation:

$$Ln(a_1) \sim \mathcal{N}(0.001, 1000) \tag{5.19}$$

$$Ln(a_2) \sim \mathcal{N}(0.001, 1000) \tag{5.20}$$

$$a_1 = exp(Ln(a_1)) \text{ and } a_2 = exp(Ln(a_2)) \tag{5.21}$$

The standard deviations of the residual error, σ_ε was considered unknown, i.e. without particular prior knowledge on its value (Eq. 5.22):

$$\sigma_\varepsilon \sim \text{Uniform}(0.5, 10) \tag{5.22}$$

5.2.4 Model parameter estimation and software

To solve the model equation system and consequently to provide estimates for the model parameters, with their credibility interval, a Markov Chain Monte Carlo algorithm was run. This technique was carried out with the Winbugs package (version 1.4.3, Medical Research Council, UK). To check the convergence of the iteration process, visual analyses (history function and Gelman and Rubin diagnostic) of three independent chains were performed. A set of 20,000 iterations were run, the first 10,000 iterations were eliminated (burn in period). No convergence problems were detected. The coefficient correlation matrix was deduced from pairwise correlation values, obtained after the discarded 10,000 iterations.

To determine the combinations of process and formulation conditions providing a given detection time (see contour plot in Result section), the set of equations 4 to 10 were solved algebraically.

5.3 Results

5.3.1 Model goodness of fit

The model (equations 5.1, 5.3-5.5, 5.7-5.10) was fitted to the dataset, using a Bayesian approach. The model outputs provided adjusted values for both strains, and for both censored data and non censored data. Figure 5.1 is an attempt to get an overview of the results, however it is imperfect as the censored data (empty symbols) could have been depicted anywhere on the x-axis from the value 4.1 onwards. For short detection time values, ln(detection +1) < 1.5 corresponding to detection < 3.5 days, the model slightly over-estimated the detection times. Nevertheless, for REPFEDs for which the shelf-life is around 4 weeks, the values below 1 week are of limited interest. Overall, for observed and censored data, the model provided a correct fit (i.e. no bias, realistic parameter estimates (e.g. $pH_{\min}$), limited correlations and sufficiently precise), for both strains, the residual model error standard deviation (ε) was equal to 1.36.

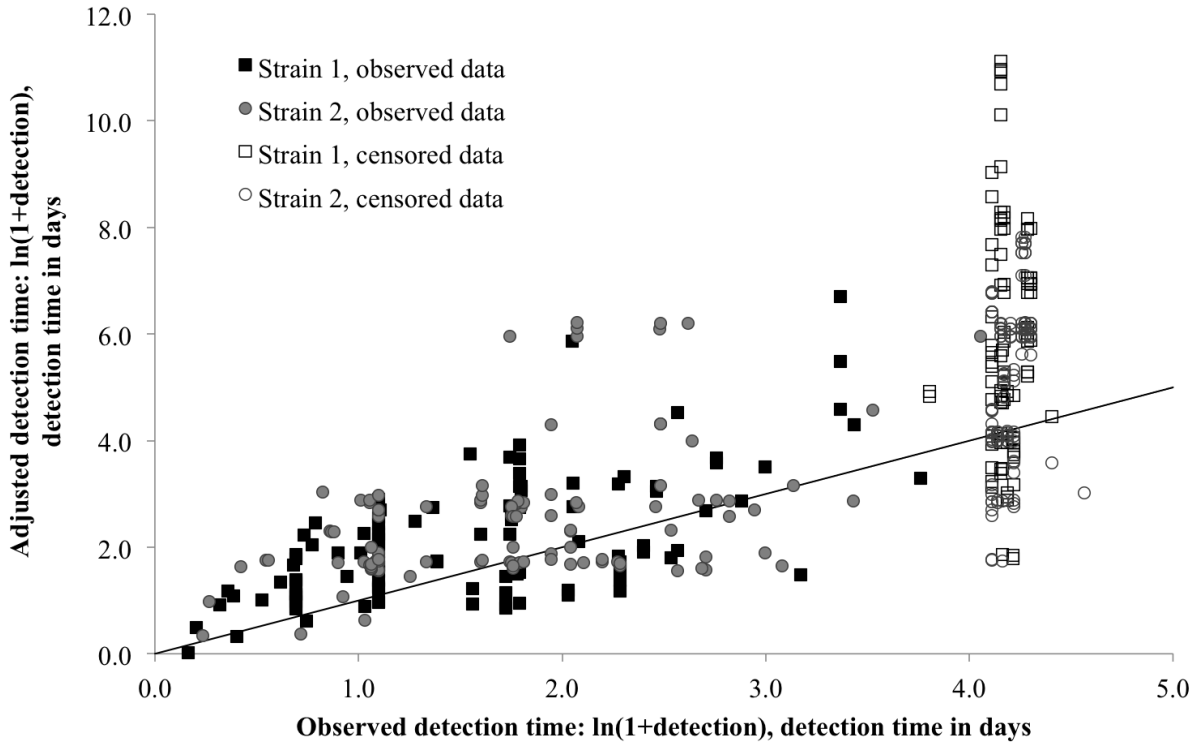
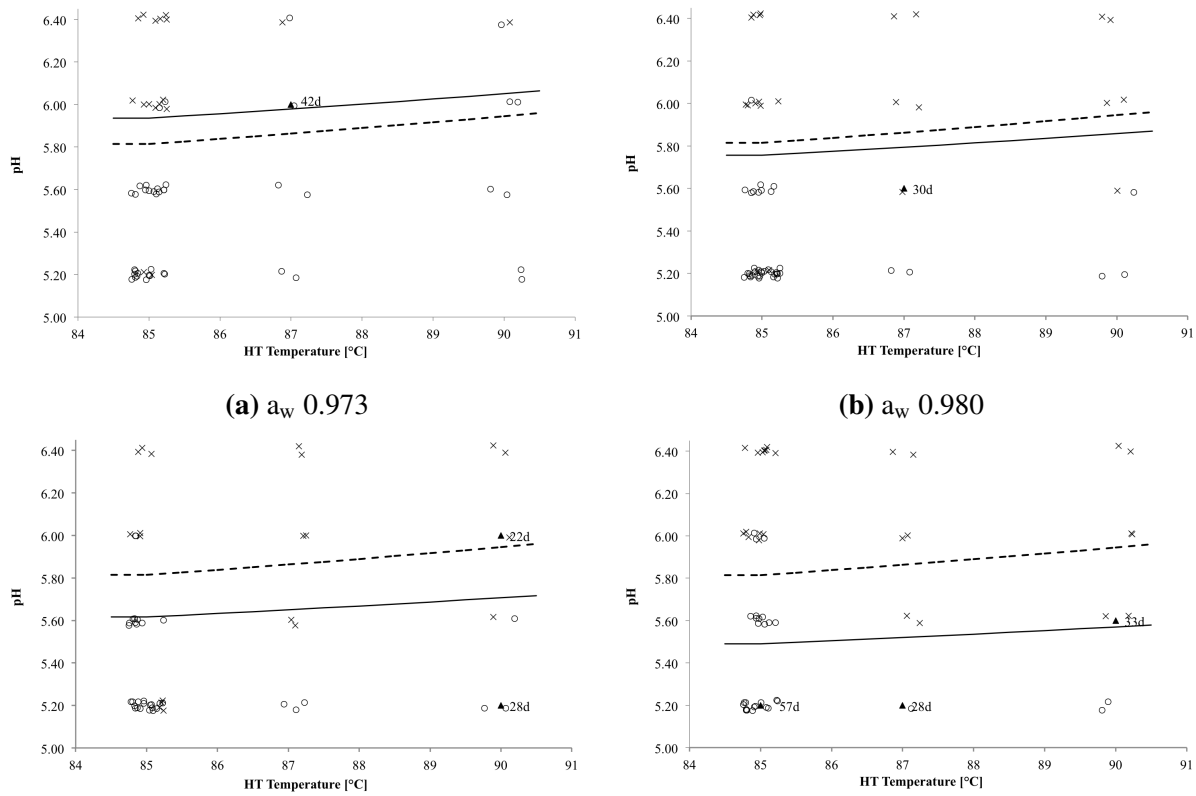


Figure 5.1: Adjusted vs. observed logarithm of detection time + 1, in days, for both strains. Values and Censored values.

Before applying the model to design process and formulation conditions suitable for REPFEDs, it was decided to scrutinise the model predictability. That was done with the factors heat-treatment and pH as representative of process and formulation settings. The procedure was set up as follows. From the model, the pH values required to have a detection time of 30 days (same order as REPFED product shelf-life) were calculated for various conditions of heat-treatment temperature and reported in a 2D-plot. This calculation split the experimental domain into two parts: on one hand combination of heat treatment and pH where the detection time was expected to be shorter than 30 days and on the other hand combinations of heat treatment and pH where the detection time was expected to be longer than 30 days. This representation is relatively similar to the Growth/No-growth interface plots in chapter 4. Then, for each combination of chilled storage temperature (10°C) and a_w (0.973, 0.980, 0.987, 0.995), the observed data were plotted (Figure 5.2). At stressful conditions but relatively high a_w ($\geq$ 0.98), strain 2 exhibited longer detection times than strain 1 (Figure 5.2b, 5.2c and 5.2d). On the other hand, at a_w 0.973, strain 2 had shorter detection time than strain 1 (Figure 5.2a). This difference in strain detection time was due to the difference in a_w effect on strains (Eq. 5.7): the a_w did not have a significant effect on the detection time of strain 2 (in Figure 5.2, the dotted line is always at the same place). The percentage of fail-dangerous predictions (detection time predicted longer than 30 days while observed detection time shorter) was 3%, 6%, 14%, 4% for a_w of 0.973, 0.98, 0.987, 0.995, respectively. These percentages are reasonable for model predictions, except for a_w 0.987 (although there was no obvious reason for a lack of model predictability at this a_w value).

To further evaluate the model predictability, additional data not used to build the model were compared to the model predictions (Table 5.3). The new conditions tested were as follows: low storage temperature (8 or 10°C), low pH (5.2 and 5.6), low a_w (0.973 and 0.98) and no heat-treatment (but only spores were inoculated to keep everything else comparable). The results confirmed that *B. cereus* detection time depended on storage temperature, pH and a_w and that a stressful condition such as 10°C, pH 5.2 and 0.98 leads to a detection time over 60 days. The results indicated also that the time-to-detect growth model, although developed for heat-treated spores may be applied to non-heated spores under a combination of stressful conditions of chilled temperature, pH and a_w. The extrapolation is feasible as the model predictions are systematically on the safe-side (predictions similar or shorter than the actual detection time). In any case, to use the model at an operational level further validation, for example by challenge-testing, is recommended.



(a) a_w 0.973 **(b)** a_w 0.980

(c) a_w 0.987 **(d)** a_w 0.995

Figure 5.2: pH versus heat-treatment temperatures, applied for 33 min, at 10°C, for a_w of 0.973 (A), 0.98 (B), 0.987 (C) and 0.995 (D). Observed values were deliberately spread around their values to get all them visible: detection time <30 days (x) and censored values with detection time > 60 days (•). When the observed detection time was close to 30 days, values were reported with a different symbol (▲) and labelled. Predicted detection time at 30 days for strain 1 (——) and strain 2 (- - - -).

Table 5.3: Comparison between model predictions and experimental results obtained with non-heated spores (data not used for building the predictive time-to-detect growth model)

Storage temp.[a]	pH	a_w	N° of experiments	Results	Model predictions (based on strain 1)
8	5.2	0.973	2	>60 d	>60 d
8	5.2	0.98	2	>60 d	>60 d
8	5.6	0.973	2	>60 d	>60 d
8	5.6	0.98	2	>60 d	35 d
10	5.2	0.973	4	>60 d	>60 d
10	5.2	0.98	4	>60 d	>60 d
10	5.6	0.973	4	>60 d	20 d
10	5.6	0.98	4	[15.9 - 55.8][b]	12 d

[a] Storage temperature (°C), [b] Range of observed lag times (days)

5.3.2 Model parameter estimates and comparison between the two strains

The model parameters with their mean, median and 95% credibility interval are given in Table 5.4. Estimated value of the coefficients $T_{\min,1}$, $T_{\min,2}$, $pH_{\min,1}$, $pH_{\min,2}$, $a_{w,\min,1}$ are close to biological minimal values where growth is observed for *B. cereus* (EFSA, 2005b). The estimated $T_{\min,1}$ and $T_{\min,2}$ in one hand, and $pH_{\min,1}$ and $pH_{\min,2}$ in the other hand differed slightly, although this difference was not significant as there was an overlap in the 95% credible interval. With the multiplicative Gamma model structure, this difference could be interpreted straightforwardly: strain 2 was less sensitive to chilled storage temperature or acidic conditions than strain 1 ($T_{\min,2}$ lower than $T_{\min,1}$, $pH_{\min,2}$ lower than $pH_{\min,1}$). However, when taking into account the constants a_1 and a_2 in the model predictions, overall longer detection times were predicted with strain 2 (for $a_w \geq 0.98$).

Table 5.4: Estimated model parameters: mean, median and 95% credibility interval

Model parameter	mean	st. dev.	2.50%	median	97.50%
a_1	0.68	0.12	0.44	0.68	0.9
a_2	1.11	0.2	0.72	1.11	1.52
$HT_{\max}$	120.3	3.1	114.2	120.3	126.4
$T_{\min,1}$	4.94	0.84	3.01	5.05	6.26
$T_{\min,2}$	3.36	1.69	-0.23	3.42	6.43
$a_{w,\min,1}$	0.923	0.016	0.881	0.927	0.944
$pH_{\min,1}$	4.48	0.11	4.25	4.49	4.65
$pH_{\min,2}$	4.17	0.14	3.83	4.19	4.4
pH_{opt}	6.23	0.25	5.94	6.16	6.99
σ_ε	1.36	0.07	1.23	1.35	1.5

Many REPFEDs are formulated at high a_w: beef stew 0.991, mashed carrots and potatoes 0.994, beans and onions 0.993, ratatouille 0.991, cow tongue 0.986, mash potato and fish 0.993, spaghetti carbonara 0.994, chicken curry 0.995, etc. At a_w values of 0.98 or higher, the model based upon strain 1 predicted shorter detection times than the model based upon strain 2 (Figure 5.3). Consequently, the model outputs presented in the following sections are illustrated with strain 1, and the model for this strain will be used in the quantitative exposure assessment in chapter 7.

In Table 5.5, the parameter correlation matrix is given. No high correlations were noticed. The highest values observed were between the coefficients a_1 and pH_{opt} (-0.69) and the coefficients a_2 and pH_{opt} (-0.67). To verify that the detection time predictions could be derived from the

mean values of the parameters, i.e. predictions could be done with the correlations neglected, the following comparison was made. The detection times predicted with the whole probability distribution of each parameter (using Winbugs) were compared to the detection times deduced from the equations in which the parameters were set at their mean values (calculations implemented in Excel). No difference was noticed (data not shown). It was then possible to illustrate the effect of the studied factors on the detection time (sections below) and to calculate the combination of factors delivering a given detection time (last section), using the mean parameter value instead of the whole probability distributions.

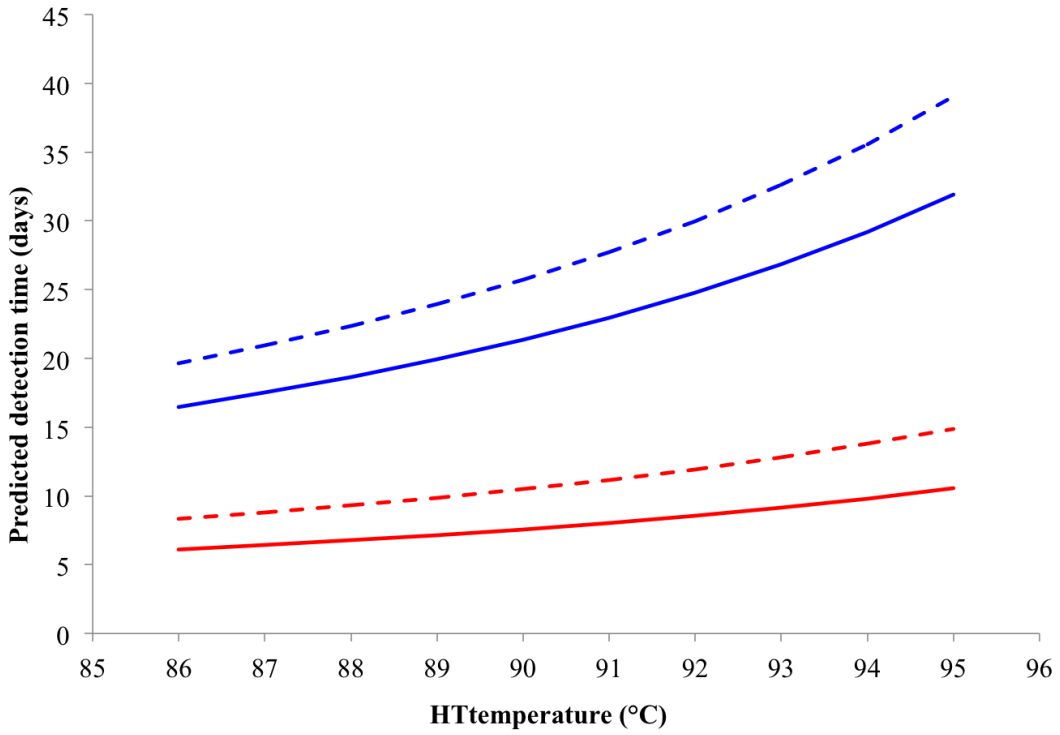


Figure 5.3: Predicted detection times versus heat-treatment temperatures for various pH conditions (pH 5.8 = dotted lines, pH 6.2 = solid lines) and for strain 1 (red) and strain 2 (blue). Heat-treatment time of 10 min, storage temperature of 10°C, a_w 0.98

Table 5.5: Model parameter correlation matrix

Model parameter	a_1	a_2	$HT_{\max}$	$T_{\min,1}$	$T\min,2$	$a_{w,\min,1}$	$pH_{\min,1}$	$pH\min,2$	pH_{opt}
a_1	1	0.56	0.06	-0.39	-0.02	-0.24	-0.56	-0.26	-0.69
a_2	-	1	0.06	0.12	-0.61	0.11	-0.28	-0.5	-0.67
$HT_{\max}$	-	-	1	-0.003	-0.02	0.17	0.12	0.08	0.07
$T_{\min,1}$	-	-	-	1	-0.01	0.13	0.24	-0.05	-0.11
$T_{\min,2}$	-	-	-	-	1	0.01	0.11	0.1	-0.004
$a_{w,\min,1}$	-	-	-	-	-	1	0.37	0.05	0.04
$pH_{\min,1}$	-	-	-	-	-	-	1	0.35	0.34
$pH_{\min,2}$	-	-	-	-	-	-	-	1	0.39
pH_{opt}	-	-	-	-	-	-	-	-	1

5.3.3 Effect of pH, a_w, chilled storage temperature and heat-treatment

The data analysed in this study showed that a combination of stressful conditions significantly extended the detection time. For example, at 8°C, 13 combinations of heat-treatment, pH and a_w were identified that lead to detection times longer than 60 days (Table 5.6). For these 13 conditions (39 data), the model predictions were in full agreement (no incorrect predictions for either strain). Likewise, at 10°C, after a pasteurisation at 90°C for 10 min and with a pH of 5.8, the detection time was predicted to be 11 days in a product at high a_w (0.99) but longer than 50 days if the a_w dropped to 0.97 (illustration in Figure 5.4 with strain 1).

The detection time was predicted to be significantly extended in presence of certain combinations of stressful conditions. Milder conditions, even applied in combination, were not considered sufficient to guarantee a long detection time and hence the stability of REPFED. For instance, heat treatment applied at 88°C for 1 min in a product at pH 5.6 and stored at 10°C, or a slightly stronger heat treatment (88°C for 10 min) in a product at pH 5.8 stored at 10°C (in both case, a_w 0.99), did not lead to detection times longer than 10 days (Figures 5.4 and 5.5).

The heat treatment as sole intervention was insufficient to extend the detection time more than a few days. After a heat-treatment at 90°C for 10 min, required to control non-proteolytic *C. botulinum*, in a product at pH 6.2, a_w 0.99 and kept at 10°C, the detection time was predicted to be no longer than 5 days (Figure 5.4). Although extrapolation should be executed with care, if the storage temperature remained at 10°C, and pH and a_w conditions were not stressful, the model did not predict a much longer detection time when a stronger heat treatment was applied

(either by longer heat-treatment time or heat-treatment temperature). For instance, with a heat-treatment of 95°C for 10 min, the predicted detection time was still lower than one week when pH equalled 6.2 (Figure 5.4). Likewise, the pH on its own was not predicted to inhibit the spore germination and outgrowth process for long. Even at pH 5.6, if the heat treatment is mild (88°C for 1 min), the detection time was predicted to be no longer than eight days (Figure 5.5).

The equivalence in pasteurisation settings was analysed. With a z-value of 9.6°C (Chapter 4), 90°C for 10 min is equivalent to 87°C for 21 min, and also to 95°C for 3 min. These three pasteurisation regimes were assessed to be equivalent in terms of detection time: respectively 13, 12 and 11 days in product at pH 6.0, a_w 0.98, stored at 10°C.

In conclusion, only the combination of chilled temperature (10°C or lower), acidic pH conditions (pH 5.8 or lower), sub-optimal a_w conditions (a_w 0.98 or lower) and moderate thermal treatment (e.g. 90°C for 10 min or higher) enabled extending the detection time significantly (e.g. more than three weeks) and consequently to assure product stability of REPFED. More combinations of process and formulation conditions are provided in the next section.

Table 5.6: Data collected at 8°C at various conditions of heat-treatment temperature (HT_T) and time (HT_t), pH and a_w. All these data were censored values (detection time > 60 days) whatever the strain.

HT_T [°C]	HT_t range [min]	pH	a_w	N° of experiments
85	27-38	5.2	0.973	5
87	12-25	5.2	0.973	6
85	27	5.2	0.98	1
85	33-38	5.2	0.987	3
87	12-25	5.2	0.987	5
85	27-33	5.2	0.995	3
87	12	5.2	0.995	2
87	20-25	5.6	0.973	2
85	33-38	5.6	0.98	4
85	27	5.6	0.987	2
87	20-25	5.6	0.987	3
87	12-20	5.6	0.995	3

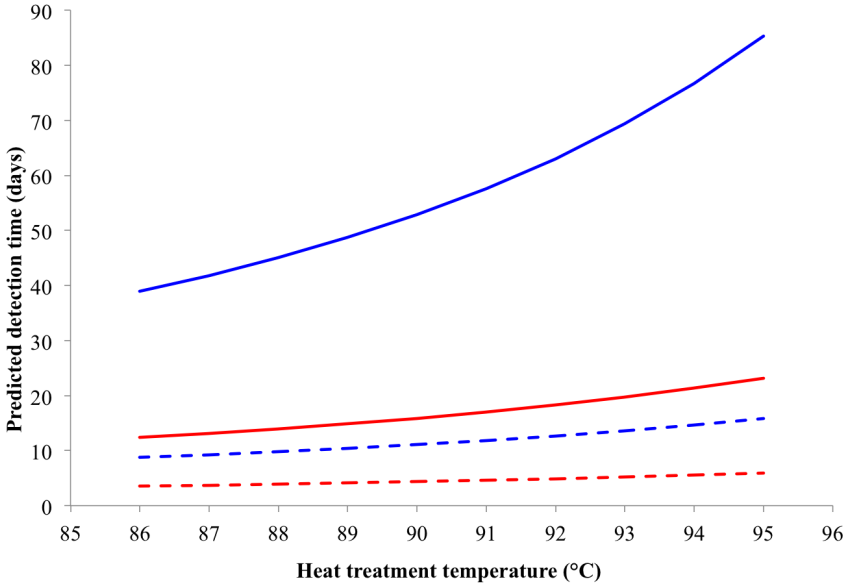


Figure 5.4: Predicted detection times of strain 1 versus heat-treatment temperatures for various conditions of pH (red = 6.2, blue = 5.8) and a_w (solid lines = 0.97, dotted lines = 0.99). Heat-treatment time of 10 min, storage temperature of 10°C.

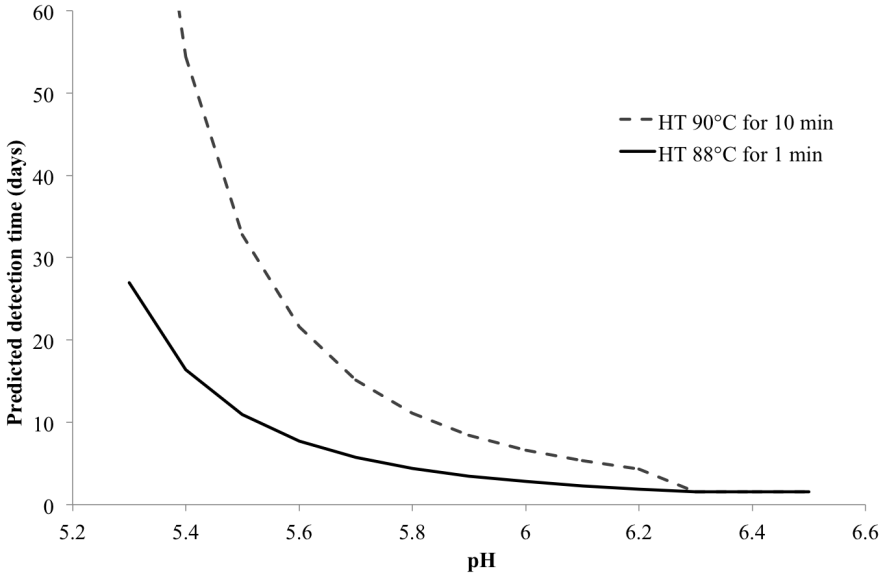


Figure 5.5: Predicted detection times of strain 1 versus pH for two conditions of heat treatment, storage temperature of 10°C, a_w of 0.99. (solid line: 88°C for 1 min, dotted line:90°C for 10 min)

5.3.4 Combinations of process and formulation conditions able to deliver a given detection time

Combinations of formulation and storage temperature delivering a given detection time can be generated directly from the equation system. In Figure 5.6 and 5.7, examples of contour plots are presented for detection times of 30 days or 50 days (with strain 1). As indicated in Figure 5.6, with a mild heat-treatment (90°C for 1 min), the detection time will be shorter than 30 days if the storage temperature is higher than 10°C unless the pH is acidic (e.g. pH 5.3 at 10°C, or, pH 5.0 at 18°C). This result means that during REPFED preparation, for example after mixing and first cooking of ingredients, the plant room temperature is too high to expect any significant detection time and thus significant spore lag time.

In Figure 5.7, various conditions of pH and a_w that enable an extension of the detection time up to 30 days, after the commonly applied heat treatment of 90°C for 10 min, are presented. During storage of products, at chilled conditions, even if the average temperature is around 6°C, there is a risk to have temperature as high as 10°C (Derens *et al.*, 2004). At this storage temperature and with a standard heat treatment of 90°C for 10 min, pH and a_w have to act in combination to extend the detection time to 30 days: pH 6.0 - a_w 0.97, pH 5.7 - a_w 0.98 or pH 5.5 - a_w 0.99 are conditions suitable to guarantee the desired detection time (Figure 5.7).

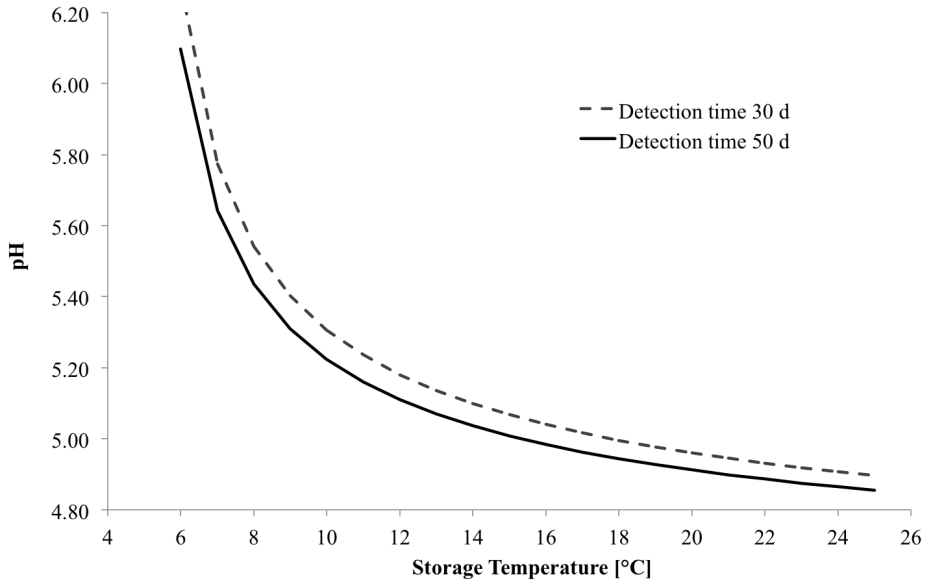


Figure 5.6: Contour plot: combination of pH and storage temperature assuring a detection time of 30 days or 50 days. Heat-treatment of 90°C for 1 min, a_w 0.99, Strain 1.

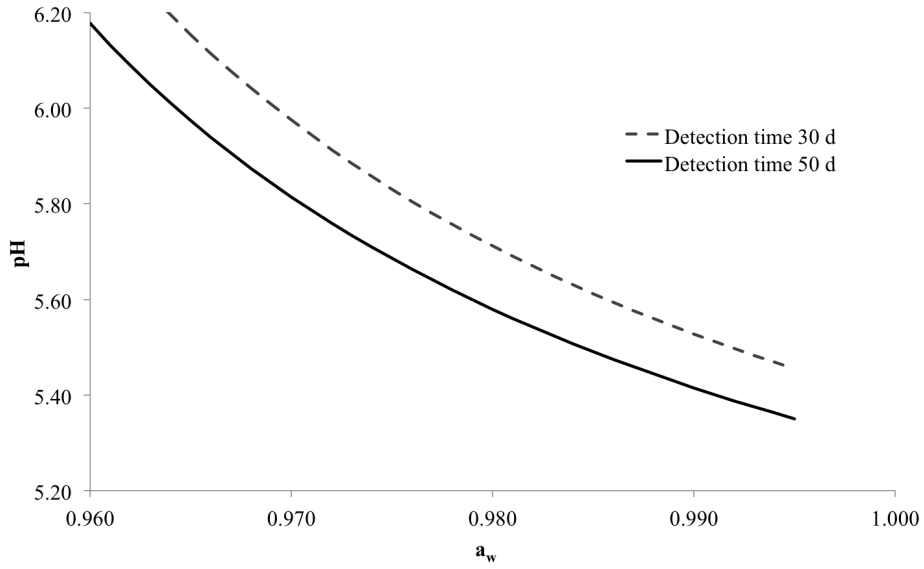


Figure 5.7: Contour plot: combination of pH and a_w assuring a detection time of 30 days or 50 days. Heat-treatment of 90°C for 10 min, storage temperature 10°C, Strain 1

5.4 Discussion

A mathematical model quantitatively assessing the effect of heat treatment, pH, a_w and storage temperature on the detection time of *B. cereus* heat-treated spores was developed. To our best knowledge, there is no such model with either detection time or lag time as response, publicly available in either literature or databases. Gaillard *et al.* (2005) developed a heat-treated *B. cereus* spore lag time, but taking into account only heat treatment and pH (experiments performed at a storage temperature of 25°C, without any variation of a_w). Modelling thermally stressed spore lag time is difficult as the biological mechanism involved is complex (Augustin, 2011). It encompasses spore damage, spore repair and cell outgrowth (Smelt *et al.*, 2008; Ter Beek *et al.*, 2011) and definitively more difficult to quantify than a vegetative cell lag time.

In the model presented here, the residual error corresponded mainly to uncertainty, first of all due to the lack of replicates in the experimental design, i.e. with a higher number of replicates it would have been smaller. This uncertainty has various sources. In our case, the statistical response analysed was not exactly the lag time before germination but the sum of two times: time required to have spore germination and outgrowth, and time to have the subsequent vegetative cells growing up to a detectable level. In addition, the detection time could vary as function of the experimental design (the wells were not systematically checked every day). The uncertainty was

also due to the large set of censored data, imprecise by definition. Beside uncertainty linked to the data, there was as well a non negligible uncertainty due to the model. It might be explained by the model constraints: based on monotonous and algebraically simple functions. A linear model with a polynomial function of degree three or more might have provided a better fit. That was not the choice made here, for reason of practicality in future use, i.e. for designing process and formulation delivering a given detection time. In addition, the model uncertainty might be due to the use of existing data, not generated in the first place to develop a time-to-detect growth model, but a growth/no-growth interface model. In the latter case, the experimental design is set up around the interface and then automatically many censored data are generated (no-growth interface). A better method would have been to use actual growth curves place on plate-counting. However, this method is much more labour-intensive in comparison with the OD measurements from chapter 4, which means that fewer combinations can be tested within the same timeframe. Additionally, the use of classical methods would require the use of larger volumes (±100-200 mL) because some medium is removed with each measurement. However, preliminary experiments showed that heating these volumes in a controllable and reproducible way was not possible with the available technology and funding. Nevertheless, developing predictive models using existing data is still a valuable desk-exercise to attempt as it is less costly than generating data from scratch.

On the other hand, the phenomenon undergone by heat-treated *B. cereus* spores before germinating and recovering in stressful conditions has been reported as complex and naturally variable (Hornstra *et al.*, 2009). Likewise, Stringer *et al.* (2011) studying the lag time of *C. botulinum* spores at a single cell level, emphasised that the variability in individual spore responses was high. This spore response variability, which is inherently present in the data, comes on top of the uncertainty and is also included in the residual error. This variability, which increases after heat treatment, is probably the reason so few models are available for heat-treated *B. cereus* spores (Table 1.4, p.28).

The model developed in our study includes the two strains within a single structure, the residual error was considered to be the same whatever the strain. Such hierarchical non linear model is easy to set up in a Bayesian framework. Another advantage of a Bayesian approach is the possibility of combining experts' opinion and data in the model parameter estimation process. In our study, experts were asked to set the model parameters either to a single likely value (HT_{opt}, T_{opt} and $a_{w,\text{opt}}$) or to an informative probability distribution ($T_{\text{min},1}$, $T_{\text{min},2}$, $pH_{\text{min},1}$, $pH_{\text{min},2}$, pH_{opt}, $a_{w,\text{min},1}$ and HT_{max}). More generally, Bayesian inference has previously been used successfully in food microbiology, for instance to build hierarchical model (Busschaert *et al.*, 2011; Crépet

et al., 2009; Membré *et al.*, 2011) or growth models (Jaloustre *et al.*, 2011; Pouillot *et al.*, 2003).

The effect of heat-treatment on *B. cereus* spore injury has been already reported (Faille *et al.*, 1997). Likewise, an increased inhibitory effect (longer lag time) on germination of *B. subtilis* spores after heating at 90°C for 5 min compared to spores that were not heated, has been established (Ciarciaglini *et al.*, 2000). With non-proteolytic *C. botulinum* spores, it has been reported that due to thermal injury, the time for the spores to recover is prolonged (Peck *et al.*, 1995). Interestingly, in the studies mentioned above, the combination of effects due to heat-treatment and pH is not mentioned while in our study, the heat treatment as sole intervention measure was not sufficient to guarantee stability of products. On the other hand, with *C. botulinum*, Stringer *et al.* (2011) came to the same conclusions as ours, the authors emphasised that the spore lag time depended on both the historic treatment of the spores and the prevailing growth conditions.

Contour plots are useful graphical tools to visualise the combinations of process and formulations delivering a given detection time; as such, contour plots can be assimilated to decision tools for R&D product designers and risk assessors. In this study, contour plots were easy to generate since the mathematical expressions chosen to define the model were monotonous and had algebraic solutions. These mathematical constraints were added deliberately in the model development to obtain an easy-to-use predictive model helping in food product design. The drawback of this choice was that the constraints could penalise the model accuracy. A Gamma multiplicative structure, although modified, seems appropriate for such model development. Gamma models have already a long history of successful applications in the food safety domain as they have been extensively used for describing the effect of temperature, pH and a_w and preservative agents on the growth rates. In such a case, a square root transformation of the growth rate is often performed to stabilise the variance (Ross & Dalgaard, 2004), meaning that the multiplicative Gamma structure is maintained despite the transformation:

$$Y = \hat{Y} + \varepsilon, \text{ with } \hat{Y} = f(T) \cdot g(pH) \cdot h(a_w) \cdot \ldots \tag{5.23}$$

Such multiplicative Gamma structure has highly valuable advantages over other model structures: parsimony in parameters, facility of interpretation of the relative impact of each inhibitory factor, and flexibility in adding new inhibitory factors to the model.

There are not many applications of the Gamma structure on lag times. So far, in predictive microbiology, the lag phase has often been interpreted as a period during which a certain amount of work has to be done to enable subsequent growth (Baranyi & Pin, 2004; Robinson *et al.*, 1998). The work-to-be-done can be written as the multiplication of the growth rate and the lag time (Koutsoumanis, 2001). It has often been assumed that the work-to-be-done is constant, whatever

the environmental conditions (Ross & Dalgaard, 2004) and then, only the growth rate has been modelled (the lag time could be derived from the growth rate model). Here, the statistical response was "ln (detection + 1)", which is close to "ln(lag+1)" as the detection time was for a large part explained by the lag phase (the inoculum was high). The logarithm transformation of the lag time is rather frequent in food microbiology. For instance, in one of the first key papers in predictive microbiology, Zwietering *et al.* (1994) evaluated various data transformation and concluded that the logarithm transformation for lag times values was recommended. From a large set of data collected on *L. monocytogenes*, Augustin & Carlier (2000) predicted the lag time after a log transformation. Working with *B. cereus*, in an attempt to predict the lag time after a heat treatment, Gaillard *et al.* (2005) suggested a simple model, based on a logarithm transformation. Likewise, Smelt *et al.* (2002) working on *Lactobacillus plantarum* cells injured by a heat treatment, modelled the lag time distribution after a logarithm transformation.

The structure of the time-to-detect growth model applied to heat-treated spore, developed here is close to a multiplicative one: $Y = \hat{Y} + \varepsilon$ and $\hat{Y} = f(T) \cdot g(pH) \cdot h(a_w) \cdot \ldots - 1$. The results are satisfactory enough to consider that despite the log transformation, the gamma multiplicative structure was still relevant. However, the extra parameter "-1" may lead to negative detection time when the factors storage temperature, pH, a_w and heat treatment are all together near their optimal values. Development of heat-treated spore lag time models should increase in the near future.

5.5 Conclusions

In conclusion, a mathematical model predicting the time-to-detect growth of *B. cereus* heat-treated spore has been developed. The heat treatment was applied after spore inoculation to build a model which can be included in a quantitative risk assessment model mimicking a production process from raw materials to product consumption. Indeed, the spores are likely to be present in the raw material and then mixed with the product preparation, at a given a_w and pH, before packaging and pasteurisation.

The model describes the effect of thermal process and product formulation (pH and a_w) on *B. cereus* behaviour, at chilled conditions. When building the model, we deliberately chose to inoculate the two strains at a high level to obtain a detection time relatively close to the lag time. When applying the time-to-detect growth model to a REPFED in order to determine the end-product pack contamination level, it will be necessary to combine the post-process contamination level,

the detection time and the growth rate, to calculate the level in the end-product. In REPFEDs, the post-process contamination level is likely to be low (Carlin *et al.*, 2000b; Del Torre *et al.*, 2001), and knowing that a low spore concentration decreases the probability of germination (Abee *et al.*, 2011; Caipo *et al.*, 2002; Peck & Stringer, 2005), the model developed in our study might slightly under-estimate the actual lag time in REPFEDs. The model is incorporated in the quantitative exposure assessment in chapter 7 to assess the risk related to *B. cereus* in REPFEDs. It can also be used on its own, to suggest combinations of process and formulation delivering a given detection time.

Although the model in this chapter is included in the exposure assessment in chapter 7 and not the growth/no-growth (GNG) model from chapter 4, both models have their merits (and downsides). The GNG model has the benefit that it is easy to interpret (e.g. "probability of growth with this pH, aw and after 30 days is x% and based on the distance between the chosen combination and the GNG boundary it will probably remain safe during shelf life"). However, the GNG model is more difficult to use for predicting the time after which growth occurs. By contrast, the model presented in this chapter can easily predict the time to growth, but because this value is given with uncertainty it is more difficult to assess if a product will stay safe during the shelf life (which is easier to estimate on the distance to the GNG boundary).

Chapter 6

Consumer behaviour related to the consumption, storage and preparation of REPFEDs

Redrafted after:

Daelman, J., Jacxsens, L., Membré, J. M., Sas, B., Devlieghere, F. & Uyttendaele, M. (2013d). Behaviour of Belgian consumers, related to the consumption, storage and preparation of cooked chilled foods. *Food Control*, 34(2):681–690

Summary

The consumer exposure to pathogenic microorganisms due to the consumption of REPFEDs is both influenced by the industrial production process and by the behaviour of the consumer. A consumer survey was organised and conducted to assess the consumption frequency, storage time, reheating practices, perception of 'use by' date and respect for the product's 'use by' date. The survey was conducted at a local food fair in Belgium with 874 respondents correctly completing the questionnaire. Over three quarters had consumed at least one REPFED over the last year. Nine out of ten consumers were able to give an acceptable estimate of the shelf life of REPFEDs (e.g. less than four weeks). By contrast, only half of the consumers fully respected the 'use by' date as indicated on the packaging. In addition, only half of the consumers fully complied with the reheating instructions on the label. To determine the distribution of the time a REPFED spends in a consumer fridge, the consumers were asked how frequently they bought REPFEDs and how they stored them. This information was used to construct a time-to-consumption (TTC) distribution. This TTC demonstrated that approximately one fifth of REPFEDs were consumed on the day of purchase; about half were consumed within two days of purchase, 75% within four days and over 90% during the first week. The TTC distribution was used in the exposure assessment in chapter 7, to estimate the time of home storage.

6.1 Introduction

Several studies have pointed out that the microbial contamination of *B. cereus* and *C. botulinum* microorganisms in REPFEDs is characterised by low prevalence and low concentrations and that the microbial risk of these products is small when stored at the correct temperature and within proper time periods (Carlin *et al.*, 2000a; Daelman *et al.*, 2013b,c; Nissen *et al.*, 2002). But it has also been established that consumers do not always respect indicated instructions on time and temperature of storage or preparation of refrigerated foods (Nauta *et al.*, 2003; Sampers *et al.*, 2012). For example the temperature of 20 to 35% of domestic refrigerators temperatures in Europe has been reported to exceed 8°C (EFSA, 2007). This type of temperature abuse, as well as neglecting the 'use by' date which is indicated on the package, may render a product microbial unsafe for human consumption. It is therefore necessary to include consumer behaviour in any microbiological exposure assessment about REPFEDs (Nauta *et al.*, 2003; Sampers *et al.*, 2012).

The notice of including consumer behaviour in assessing the safety of foods is also included in the EU regulation on microbiological criteria for foodstuffs (Anonymous, 2005). Under this regulation food business operators must ensure that the food safety criteria applicable throughout the

shelf life can be met under reasonably foreseeable conditions of storage and use. For *L. monocytogenes* some recommendations on this approach to simulate transport, retail and consumer storage are included in the technical guidance document on challenge testing used by the EU community reference laboratory (EU CRL for *Listeria monocytogenes*, 2008) or in the AFNOR for shelf life testing (AFNOR, 2010). For temperature abuse at the consumer phase it is suggested to use either a temperature justified by detailed information (75^{th} percentile of the observations of home refrigeration temperatures for the proper country), or if no such data is available, to use 8°C and 12°C. For storage time it is supposed that products are kept at consumer phase for 1/3 or 2/3 of their indicated shelf life. However, it is highly unlikely that all REPFEDs are stored for their entire shelf life and consumed on the last day of their shelf life. Storage-times for various products (e.g. smoked fish, ready-to-eat foods, cold sliced ham) in consumer fridges have been reported. These were either based on expert opinion (Garrido *et al.*, 2010; Nauta *et al.*, 2003) or on consumer surveys (Pouillot *et al.*, 2010).

The first objective of this study was to gain actual data about consumer attitudes towards REPFEDs. Therefore, a survey was performed at a food fair in Ghent (Belgium), inquiring about the following items: frequency of purchase, home storage time, respect for 'use by' date and implementation of the reheating guidelines on the label. The second objective was to quantify this behaviour to determine the impact of consumer behaviour with REPFEDs on their exposure to *B. cereus* (Chapter 7).

6.2 Materials and methods

6.2.1 Questionnaire

To gain insight in the behaviour of Belgian REPFED consumers a questionnaire was developed. During the development of the exposure assessment for *B. cereus* in REPFEDs (chapter 7), the primary sources of uncertainty at consumer level were listed. Three items were selected: (i) storage time at consumer level, (ii) respect for the shelf life (i.e. how many products are consumed after the 'use by' date) and (iii) compliance to reheating guidelines. Since the frequency of consumption determines the weight of an answer in the analysis, this topic was also included in the questionnaire. To prevent ambiguity in the questions and to be as comprehensive as possible, several preliminary versions of the questionnaire were drafted and subsequently tested by the personnel of the Laboratory of Food Microbiology and Food Preservation (LFMFP) at Ghent University. The final questionnaire was presented to visitors of a food fair in Ghent (17-20 May 2012). Participants were offered an incentive (drink, chocolate) for filling out the question-

naire. To enable more people to take part in the same time period and to prevent errors in data processing, participants completed a questionnaire using a laptop and a web-based application (survey monkey). The questionnaire was completed under supervision, which allowed a more correct data gathering (e.g. no young children or groups) and gave the participants the possibility to ask clarification if needed. The full questionnaire, translated from Dutch, including possible answers is listed in Table 6.1. Text between brackets in italic was not visible on the questionnaire (e.g. skipping questions).

6.2.2 Data processing

Incomplete questionnaires, irrespective of the number and the nature of the question(s) left unanswered, were removed from the database. The answers to question two, five and six were recoded to acceptable/unacceptable to facilitate the discussion (Table 6.1). Correlations were evaluated using the non-parametric Kendall-tau rank correlation coefficient (SPSS 20.0, IBM) and are listed in Tables 6.4 and 6.5.

Table 6.1: Questions and answers of the survey. Text in *italic* was not visible to the participants.

N°	Question	Answers
1.	Have you ever bought a ready-to-eat or cooked chilled food? By cooked chilled food, we mean meals that you store in the fridge and reheat in the (microwave) oven before eating. Below are some examples of ready-to-eat foods? Be careful, we are not enquiring about frozen meals. *(Participants were shown four images of commercial REPFEDs in their packaging)*	a. Yes *(Go to question 2)* b. No *(Go to question 7)*
2.	Do you have an idea of the shelf life of cooked chilled foods? *Recoded for discussion:* *a and b = acceptable* *c, d and e =unacceptable*	a. Less than 14 days b. 2-4 weeks c. 2-6 months d. longer than 6 months e. I have no idea
3.	How frequently do you buy cooked chilled foods?	a. Almost every day (5-7 days a week) b. 2-4 times a week c. Once a week d. 3-5 times a month e. Now and then (once a month) f. Seldom (once a year) g. Never *(Go to question 7)*

Continued on next page

Table 6.1 – continued from previous page

N°	Question	Answers
4.	When you buy a cooked chilled food, how do you, generally, store this product before eating it? Multiple answers possible.	a. In the fridge - 0 days (buy & eat on the same day) b. In the fridge - 1-3 days c. In the fridge - 4-7 days d. In the fridge - up to two weeks e. In the fridge - more than two weeks f. In the freezer - I keep these products in the freezer and defreeze it on the day I will eat it. g. In the freezer - I keep these products in the freezer and defreeze it 1-3 days before I will eat it.
5.	How strict do you respect the 'use by' date of a cooked chilled food? *Recoded for discussion:* *a = acceptable* *b, c and d =unacceptable*	**a. Strict**: When a RTE food has passed the 'use by' date, I will no longer eat it and throw it away. **b. Moderately**: When a RTE food has surpassed the 'use by' date by a couple of days (2-3 days) I will still eat it, but not if it's more than 3 days over the date. **c. Limited**: Even if a ready-to-eat food has surpassed the 'use by' date with more than 3 days I will still eat it. Only after . . . days I will no longer eat it and throw it away. (Please specify the number of days) **d. I don't**: "If the product still looks and smells good, I will still eat it."

Continued on next page

Table 6.1 – continued from previous page

N°	Question	Answers
6.	How strict do you follow the reheating instructions on the package of a cooked chilled food? Below or some examples of such instructions e.g. - 800 watt for 4 min, stir halfway through e.g. - Putt on a plate and heat for 10 minutes at 180°C *Recoded for discussion:* *a = acceptable* *b and c =unacceptable*	**a. Completely**: "I will do all the steps listed on the package" **b. Partially**: "I will respect the time and the microwave power setting (Watt), but I will not stir the product halfway through." Or "I will respect the time, but don't know how to change the power setting (Watt)." **c. I don't**: "If it is warm enough to eat, that is good for me."
7.	What is your gender?	a. Male b. Female
8.	What is your age?	a. <18 b. 18-25 c. 26-30 d. 31-40 e. 41-50 f. 51-60 g. 61-70 h. >70

6.2.3 Construction of time-to-consumption distribution

The time during which a product is stored in a consumer fridge is of significant importance to assess the exposure to psychrotrophic pathogens. To this end, a time-to-consumption (TTC) curve was constructed. The TTC curve is determined based on a combination of three probabilities: (i) the frequency of purchase, (ii) the storage time in the consumer fridge and (iii) the respect for the 'use by' date. The Kendall tau-c statistic was used to determine if the probabilities were correlated (Table 6.4 and 6.5). Since this was the case, conditional probabilities were used. A schematic representation of these probabilities is given in Figure 6.1, the equations, variables and examples are given in Table 6.2 and all the necessary data to reproduce the calculations is given in Table 6.3.

1. The probability that a product is consumed by someone with a given frequency of purchase: P(*frequency*). This depends on the frequency of purchase (question 3). Daily consumers will consume more products than consumers who buy only one product per year.

2. The probability that a consumer respects or disrespects the shelf life (question 5) given this consumer's frequency of purchase: P(*respect* | *frequency*). This probability will determine if a product that exceeds the 'use by' date is still consumed.

3. The probability that a consumer has answered a certain time in response to question 4, given this consumer's respect for the 'use by' date and this consumer's frequency of purchase: P(*answer* | *respect* | *frequency*). This will finally determine the time a product spends in a consumer fridge.

First, the probability that a product is consumed by a certain type of consumer, depends on (i) the frequency of purchase of REPFEDs by the consumer (question 3) and (ii) the number of consumers in that group (i.e. with that frequency of purchase). Because some answers to question 3 actually contain multiple frequencies (e.g. 2-4 times per week), these were split up (e.g. 2, 3 or 4 per week), with $x_{freq,j}$ the number of REPFEDs consumed in a year for possibility j of that answer. Using this information, the probability that a REPFED will be consumed by someone with a given frequency of purchase (P(*frequency*)) can be inferred using equation 1 (Table 6.2). An example for consumers who consume REPFEDs two to four times a week is given in equation 2.

The second probability determines if a consumer will consume or throw away a product once it has exceeded the 'use by' date. Given the correlation between frequency of purchase and respect for the 'use by' date and between frequency of purchase and the storage time (section 6.3.6 and

Table 6.5), conditional probabilities were used. P(*respect* | *frequency*) is the probability that a consumer with a given frequency of consumption (e.g. 2-4 a week) has a certain respect for the 'use by' date (e.g. strict). This probability was calculated using equation 3 with the answers of question 5. An example of the calculation is given in equation 4 (Table 6.2).

The third probability, how long a consumer stores a product (question 4), was determined based on the responses given to question 4 and was correlated with both the frequency of purchase (question 3) and respect for the shelf life (question 5). P($answer \mid respect \mid frequency$) is the probability that a consumer with a given frequency of consumption and a given respect for the 'use by' date (e.g. 2-4 a week and strict) has given a certain answer to question 4 (e.g. 1-3 days in the fridge). Because storage in the freezer does not allow microbial growth, answers *a* and *f* (0 days fridge/freezer) and *b* and *g* (1-3 days fridge/freezer) essentially corresponded to the same behaviour: 0 or 1-3 days in the fridge respectively. Because no upper limit had been set for response *e* (more than 14 days), the upper limit was fixed at a high value of 30 days past the 'use by' date. The probability that a consumer used a certain storage time, given his frequency of purchase and respect for the 'use by' date, was calculated using equation 5 with the answers of question 4. An example of the calculation is given in equation 6 (Table 6.2).

The probability that a product is stored for a certain time P(*time*) (e.g. 1 day) can be calculated using equation 7. '*Answer*' is the answer of question 4 that contains the storage time wanted for calculation (e.g. if time is 4 days that the corresponding answer is c) and '*options*' is the number of possibilities within one answer (e.g. for the answer "4-7 days" this corresponds to 4). A shortened example of the calculation of P(1 day) is given in equation 8 (Table 6.2).

Using equation 1, 3 and 7 the storage time in a consumer fridge (TTC) was inferred. To account for the possibility that a product stored for longer than the shelf life may be thrown away, the calculated storage time was compared to the 'use by' date. If the storage time was shorter than the shelf life, the product was consumed. If the product exceeded the storage time, what happened with the product depended on the consumer respect for the 'use by' date (question 5). For consumers with strict respect for the 'use by' date no extra margin on the 'use by' date was used in the calculations. This means that if a product was past the 'use by' date, it was thrown away. Consumers who chose 'moderate', 'limited' or 'none' were respectively given 3 days, 7 days and 30 days of margin. This means that 'limited' respectful consumers would still consume a product if it was three days past the 'use by' date but would throw it away after four days. To test the effect of the 'use by' date, four shelf life durations were compared: 7, 14, 28 and 35 days. In the current simulation, these shelf lives comprise the time available for storage in the consumer fridge and not the complete shelf life of the product. They do not include the time

spent in internal storage, transport and retail, which are not considered. The calculations for the TTC distribution were performed in @Risk (Palisade) running 100,000 iterations.

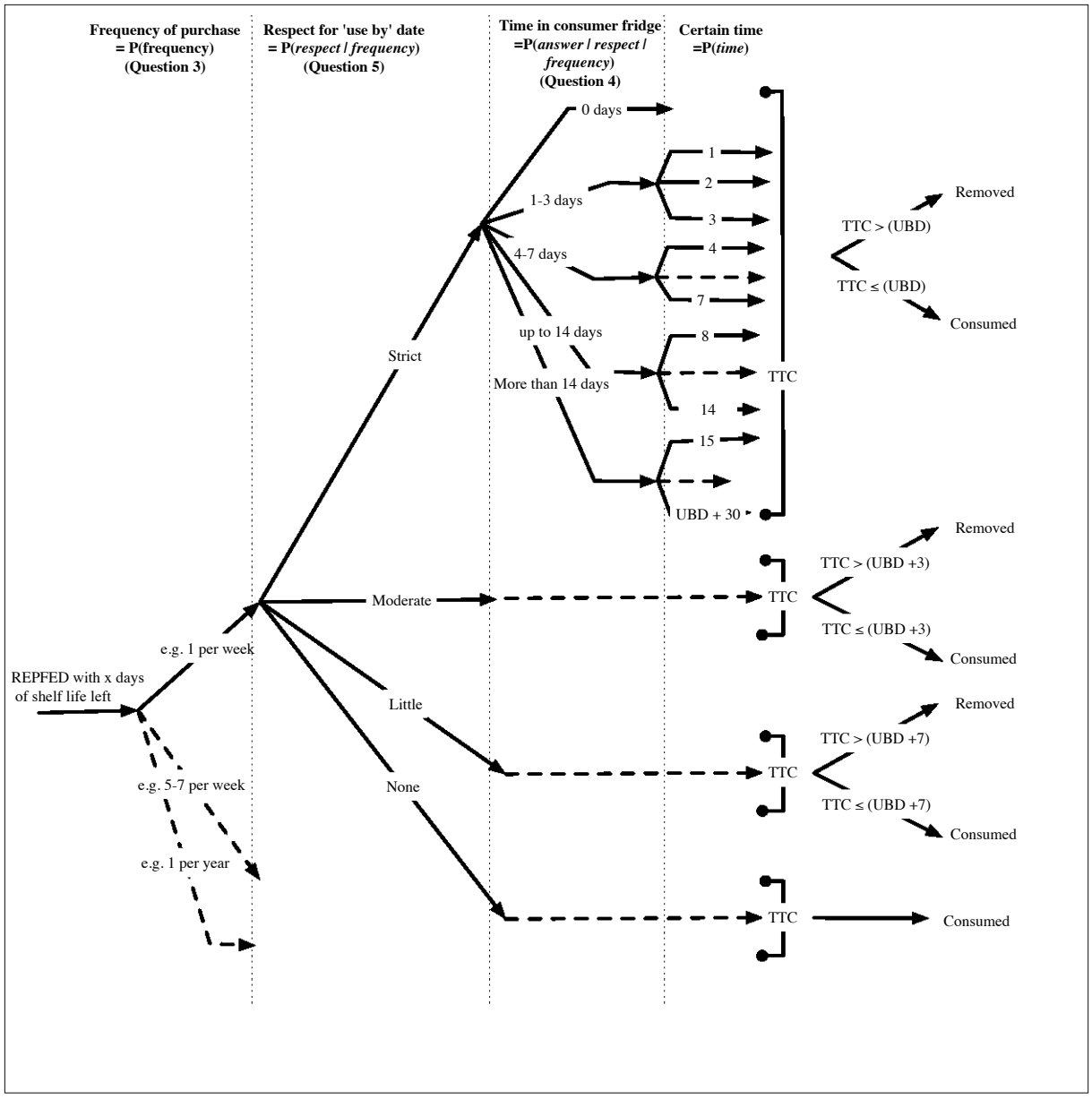


Figure 6.1: Flowchart for the calculation of the time-to-consumption (TTC). Dotted lines represent options that are identical to those above them. (UBD = 'use by' date)

Table 6.2: Equations, variables and examples used for the calculation of the 'time-to-consumption' distribution.

Probability			Equation N°
P(frequency)	Equation	$P(frequency) = \frac{n_{frequency}}{N} \cdot \text{Discrete uniform}\left(\frac{x_{freq,1}}{365}, \frac{x_{freq,2}}{365}, \ldots, \frac{x_{freq,k}}{365}\right)$	1
	Variable	$\mathbf{n_{frequency}}$: number of consumers with certain frequency of purchase , i.e. with a certain answer to question 3 (e.g. 2-4 a week) $\boldsymbol{N}$: total number or consumers (677) $\mathbf{x_{freq,k}}$: number of REPFEDs theoretically consumed in a year for each of the possible frequencies within the answer to question 3 (e.g. 2 a week, 3 a week . . .)	
	Example	$P(2-4 \quad a \quad week) = \frac{15}{677} \cdot \text{Discrete uniform}\left(\frac{(2\cdot 52)}{365}, \frac{(3\cdot 52)}{365}, \frac{(4\cdot 52)}{365}\right)$	2
P(respect | frequency)	Equation	$P(respect \mid frequency) = \frac{n_{(respect\mid frequency)}}{n_{frequency}}$	3
	Variables	$\mathbf{n_{(respect\|frequency)}}$: number of consumers with a certain respect for the shelf life and a given frequency of purchase	
	Example	$P(moderate \mid 2-4a week) = \frac{8}{15}$	4

Continued on next page

Table 6.2 – continued from previous page

Probability			Equation N°
P(answer | respect | frequency)	Equation	$P\,(answer \mid respect \mid frequency) = \frac{n_{(answer\mid respect\mid frequency)}}{n_{(all\ answers\mid respect\mid frequency)}}$	5
	Variables	$\mathbf{n_{(answer\mid respect\mid frequency)}}$: number of consumers with a certain storage time (i.e. answer to question 4) given a certain respect for the shelf life and a given frequency of purchase $\mathbf{n_{(all\ answers\mid respect\mid frequency)}}$: number of answers received for question 4 for a given respect for the shelf life and a given frequency of purchase	
	Example	$P\,(1-3\ days \mid moderate \mid 2-4 a week) = \frac{4}{11}$	6
P(time)	Equation	$P(time) = \sum_{i=1}^{6} P\,(frequency, i) \cdot \left[\sum_{j=1}^{4} \left(P\,(respect, j \mid frequency, i) \cdot P\,(answer \mid respect, j \mid frequency, i)\,\frac{1}{options}\right)\right]$	7
	Variables	***options***: number of possible storage times per answer to question 4 . E.g. for 1-3 days there are 3 options, one, two or three days of storage.	
	Example	$P\,(1\ day) = P\,(5-7\ a\ week) \cdot [P\,(strict \mid 5-7\ a\ week) \cdot P\,(1-3\ days \mid strict \mid 5-7\ a\ week) \cdot \frac{1}{3}] + P\,(2-4\ a\ week) \left[\left[P\,(strict \mid 2-4\ a\ week) \cdot P\,(1-3\ days \mid strict \mid 2-4\ a\ week) \cdot \frac{1}{3}\right] \left[P\,(moderate \mid 2-4\ a\ week) \cdot P\,(1-3\ days \mid moderate \mid 2-4\ a\ week) \cdot \frac{1}{3}\right]\right] +\ldots + P\,(once\ a\ year) \cdot [\ldots + [P\,(not \mid once\ a\ year) \cdot P\,(1-3\ days \mid not \mid once\ a\ year) \cdot \frac{1}{3}]] \approx 16\%$	8

Table 6.3: Data used to calculate the time-to-consumption. N, $n_{frequency}$ and $n_{(respect|frequency)}$: number of consumers in this group; $n_{(answer|respect|frequency)}$ and $N_{(All-answers|respect|frequency)}$: number of times this response was chosen by the consumer.

Frequency of consumption	$n_{frequency}$	Respect for UBD[a]	$n_{(respect\|frequency)}$	$n_{(answer\|respect\|frequency)}$					$N_{(All-answers\|respect\|frequency)}$
				0	1-3	4-7	8-14	>15[b]	
5-7 a week	2	Strict	2	1	1	1	-	-	3
		Moderate	-	-	-	-	-	-	0
		Limited	-	-	-	-	-	-	0
		Not	-	-	-	-	-	-	0
2-4 a week	15	Strict	6	2	4	2	-	1	9
		Moderate	8	3	4	2	2	-	11
		Limited	-	-	-	-	-	-	0
		Not	1	1	-	-	-	-	1
Once a week	92	Strict	47	5	28	14	4	-	51
		Moderate	39	6	27	13	4	1	51
		Limited	3	-	1	2	-	-	3
		Not	3	1	3	1	-	-	5
2-3 a month	123	Strict	62	12	48	12	-	-	72
		Moderate	51	13	27	17	3	1	61
		Limited	2	-	-	1	1	1	3
		Not	8	1	2	6	1	1	11
Once a month	283	Strict	159	49	99	42	7	1	198
		Moderate	105	27	70	27	6	-	130
		Limited	7	5	5	3	1	-	14
		Not	12	6	6	3	1	-	16
Once a year	162	Strict	85	36	47	13	-	-	96
		Moderate	65	14	37	16	4	1	72
		Limited	5	3	1	2	-	-	6
		Not	7	2	5	-	-	-	7
N =	677								

[a] 'use by' date

[b] 15 - 'use by' date + 30 days

6.3 Results and Discussion

6.3.1 Sample description

During the four-day period, 931 people participated in the survey on REPFEDs. Seventy-five (6.1%) questionnaires were rejected because of one or more missing answers. A total of 874 responses were considered valid. Slightly over half of the respondents were women (57.7%). The majority of the respondents (49.1%, n=429) were 30 to 60 years old, 352 people were between 15 and 30 (40.3%) and the elderly people (>60) accounted for 10.6% (n=93) of the respondents (Table 6.4). In comparison: in 2008 the Belgian population group aged 30-60 was 50.5% of the total population, 22.3% was between 15 and 30 and 27.2% was older than 60 (FPS Economy, SMEs, Self-employed and Energy, 2011).

This comparison illustrates that our sample of the population contained a relatively high amount of people younger than thirty and not enough elderly people, to completely represent the Belgian population. This over representation of younger people may be linked to the venue where the questionnaire was organised. This type of event may attract more young consumers, who are generally more mobile than elderly people. A future solution for this could be to administer the questionnaire at different events or specifically target elder people. However, given the large sample size it can still be considered a fair sample of the population, especially since younger consumers are more likely to consume REPFEDs (see section 6.3.2). It must also be noted that the results of the questionnaire are only valid for Belgian consumers and should not be lightly used for consumers in different (food)-cultures.

6.3.2 Consumption of REPFEDs

More than three quarters (77.5% n=677) of the respondents indicated they purchased at least one REPFED in the last year. These respondents were considered to be REPFED consumers. The percentage of consumers was almost identical in men (74.3% n=275 out of 370) and women (79.8%, 402 out of 504), but the percentage of consumers decreased with age (Table 6.4). If the data is divided in smaller age groups (Figure. 6.2) a trend can be distinguished, which shows a peak in the percentage of consumers between 18 and 30 and after that a steady decrease for older age groups. This trend could be expected because this type of food products are specifically designed for fast and convenient consumption and the feeling of time scarcity is larger with employed parents which are typically found in this age group (Jabs & Devine, 2006).

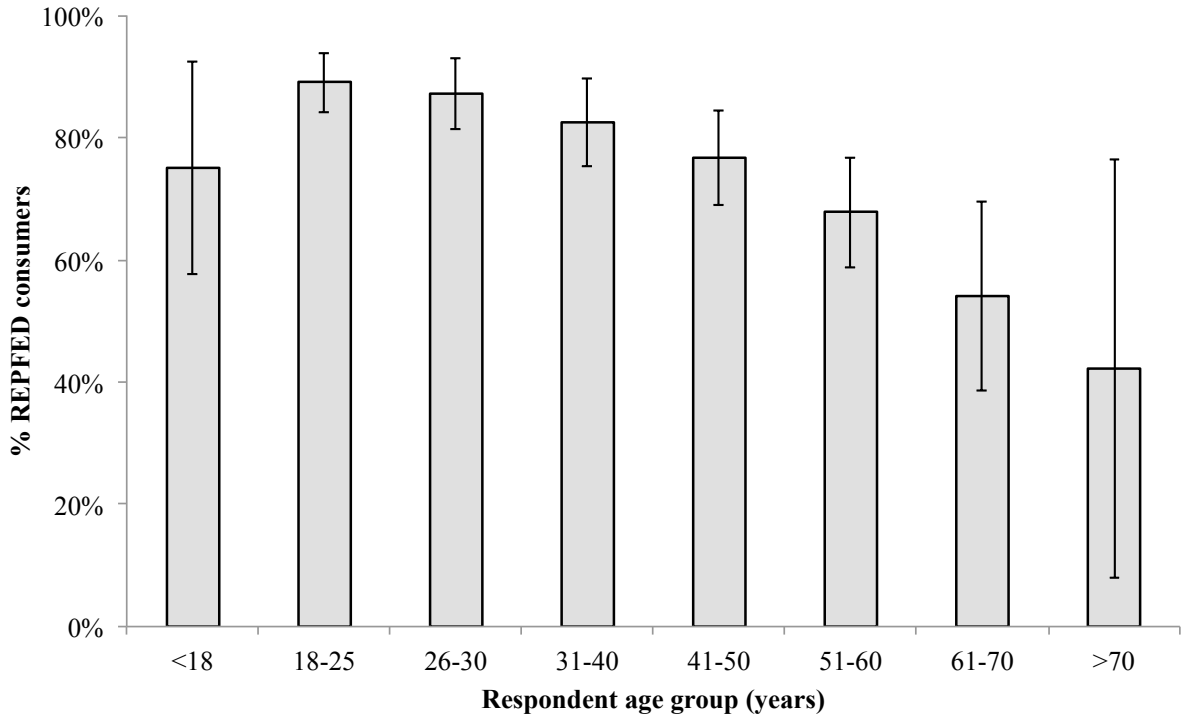


Figure 6.2: Percentage of REPFED consumers for the different age groups. Errors bars represent the 95% confidence interval.

6.3.3 Consumer perception and respect of product shelf life

Consumers (n=677) were asked to give their estimate of the shelf life of a REPFED (Table 6.1, Question 2). More than nine out of ten consumers (91.1%) gave an acceptable response of the shelf life (<14 days or 2-4 weeks), 4.6% of consumers had no idea and the remaining 4.3% thought the shelf life was more than 2 months. Women were more likely to have an acceptable perception of the shelf life than men (Table 6.5).

Consumers were also asked how strictly they observed the 'use by' date of a cooked chilled food as indicated on the package (Table 6.1, Question 5). More than half (53.3%) of the consumers strictly respected the 'use by' date. Most of the remaining consumers would still consume a product that was two to three days past the 'use by' date (39.6%) and a small group (2.5%) of consumers would still consume the product if it was more than 3 days overdue. When the latter group was asked after how many days they would no longer consume the product, the responses varied from 4 to 10 days with a median of 6 days. The remainder of the consumers (4.6%) stated that they do not take the 'use by' date in to account and would still consume an overdue product, as long as it does not smell or look bad. The percentage of consumers

reporting that they completely respected the 'use by' date is low, compared to previous surveys. Unklesbay *et al.* (1998) surveyed the attitudes and practices of 824 US college students with respect to food safety and noted that most student said they discarded food that had past its expiration date. More recently, Ergönül (2013) did a survey of 600 Turkish consumers. During the survey, 83% of the participants indicated they never consumed the food products after the 'use by' date. Not respecting the 'use by' date is risky behaviour, for example with respect to *B. cereus* or *L. monocytogenes*. Given an adequate temperature (abuse) and product formulation these pathogenic bacteria can grow to hazardous concentrations (10^{4-6} CFU/g) before spoilage becomes apparent (Choma *et al.*, 2000b; Rajkovic *et al.*, 2006; Pal *et al.*, 2008).

No significant correlation ($p>0.05$) was found between consumer respect for the 'use by' date and age or frequency of purchase. However, male consumers and consumers with an unacceptable perception of the shelf life had slightly less respect for the 'use by' date. People with less respect for the shelf life are also more likely to store the products for longer periods (Table 6.5).

6.3.4 Following the reheating instructions

Consumers were asked to what extent they complied with the reheating guidelines given on the product packaging (Table 6.1, Question 6). Nearly half of the consumers (49.9%) fully respected the reheating guidelines as indicated; the rest followed the instructions partially (36.5%) or did not follow the instructions at all (13.4%). One respondent stated that, in the case of lasagne, he preferred to consume it cold. Data analysis showed that male consumers and younger consumers were less likely to follow the reheating guidelines (Table 6.5). This practice will increase the exposure to certain pathogenic microorganisms. Although the effect of reheating is variable and will not reduce the number of spores or concentration of heat-stabile toxins, it may reduce the levels of vegetative pathogens like *L. monocytogenes* (Daelman *et al.*, 2013b; Rajkovic *et al.*, 2008) or heat labile toxins. Consumers who did not respect the reheating guidelines were also less likely to respect the 'use by' date (Table 6.5).

6.3.5 Frequency of Purchase

Consumers were asked to indicate how frequently they purchased REPFEDs (Table 6.1, Question 3). Hundred and nine consumers (16.1%) indicated they bought REPFEDs at least once a week. A small minority of these consumers (n=17, 2.5%) said they bought REPFEDs more than once a week. The majority (60%) of the consumers indicated buying REPFEDs one to five times per month, while 23.9% bought only one REPFED per year. The data showed that both age and gender affected the purchase frequency. Women and elderly people (>60) were less frequent

consumers compared to men and younger consumers (Table 6.5). This group corresponds to the target group of consumers: people seeking a fast and convenient meal and small households.

6.3.6 Storage in consumer fridge or freezer

The majority of consumers stored REPFEDs in the fridge (88.0%); the remaining consumers used the freezer (4.1%) or used both fridge and freezer (7.8%). To determine the storage duration, the responses were weighed by the number of responses given by the respondent, i.e. if a consumer had indicated two or three responses, each response will respectively count as half or a third towards the end total. The majority of consumers (73.2%) will consume a cooked chilled food within 3 days after purchasing (65.4%) or thawing (7.8%). Most of the other consumers (22.0%) will consume the REPFED within 4 to 7 days. Only a minority (4.8%) of the respondents will store REPFEDs for more than 7 days.

No significant correlation was noted between the time of storage in the fridge (after purchase or after thawing) and age or the gender of the consumer. However, there was a correlation between the storage time and the frequency of purchase. Frequent consumers were more likely to store the purchased REPFEDs for longer. In addition consumers with less respect for the 'use by' date would store the products for longer periods (Table 6.5).

Table 6.4: Kendall tau rank correlation coefficient for the different responses to the questionnaire **for all respondents** (i.e. questions 1, 7 and 8). Coefficients in bold were statistically significant (α= 0.05, → indicate the ranking of the data)

All respondents	Age young → old	Gender Men → women	Consumption No → Yes
Age	1	-0.071 (0.029)	**-0.163 (0.0)**
Gender		1	0.064 (0.06)
Consumption			1

Table 6.5: Kendall tau rank correlation coefficient for the different responses to the questionnaire **for consumers only** (i.e. questions 2-8). Coefficients in bold were statistically significant (α= 0.05, → indicate the ranking of the data).

Consumers	Idea of shelf life Acc.[a] → Not acc.	Frequency of purchase More → less	time of storage Short → long	Respect for 'use by' date strict → not	Reheating compel.[b] → don't	Age young → old	Gender men → women
Idea of shelf life	**1**	0.047 (0.073)	-0.036 (0.164)	**0.052 (0.038)**	-0.01 (0.67)	0.016 (0.526)	**-0.051 (0.023)**
Frequency		**1**	**-0.116 (0.00)**	-0.015 (0.595)	-0.012 (0.704)	**0.061 (0.033)**	**0.121 (0.004)**
Storage time			**1**	**0.060 (0.036)**	**0.060 (0.036)**	0.048 (0.095)	-0.002 (0.963)
Respect for UBD[c]				**1**	**0.120 (0.00)**	0.012 (0.70)	**-0.099 (0.011)**
Reheating					**1**	**-0.122 (000)**	**-0.085 (0.032)**
Age						**1**	.006 (0.890)
Gender							**1**

[a] Acceptable, [b] Completely, [c] 'use by' date

6.3.7 Time-to-consumption

To have a representative estimate of the storage time at consumer level, the concept of time-to-consumption (TTC) is introduced. A similar concept was introduced by Nauta *et al.* (2003) based on an expert opinion. In this study, the premise was used that 80% is consumed in the first week, 15% between one and two weeks, 5% between two and three weeks and that 5% is consumed after the 'use by' date. The goal of this distribution is double. First, it determines the number of days a REPFED will spend in a consumer fridge. This information can be combined with temperature information to assess growth and hence exposure to psychrotrophic microorganisms. Second, it enables to determine whether the product will still be consumed if it has exceeded the 'use by' date. To test the effect of the 'use by' date, four shelf life periods were compared: 7, 14, 28 and 35 days.

The results show that the majority of the products were estimated as consumed (96-99%) (Table 6.6). One fifth (19.8%) of the REPFEDs were consumed on the day of purchase, little over half of the products (52.9%) were estimated as consumed within 2 days after purchase and 93.7% was estimated as consumed within the first week after purchase. These numbers are similar to those given by Nauta *et al.* (2003) based on the percentage of packages that is consumed after the 'use by' date. They estimated that 25% is consumed within 1 day, 81% within one week and 95% within 2 weeks. They are also similar to the quartiles reported by (Pouillot *et al.*, 2010) for different RTE foods (e.g. Deli salads, soft cheese and smoked seafood). For the time to first consumption (i.e. first opening of the packages), they reported that 50% was consumed between 1 to 5 days, and that 90% was consumed within 5 to 15 days (depending on the product). For the time to last consumption (i.e. when the package is empty) they reported much longer times, with 50% being consumed after 7 to 14 days, and 90% after 12 to 46 days.

There was little difference between the TTC distribution for the four shelf life periods (7, 14, 28 and 35). However, there was a difference in the fraction of products that were not consumed and the fraction that were consumed after the 'use by' date. If the shelf life increased from 7 to 35 days, the percentage of products that was not consumed decreased from 4.3% to 0.3% and the percentage of products consumed after the 'use by' date also decreased from 2.0% to 0.1%. This is considerably less than the 5% estimated by Nauta *et al.* (2003), but slightly more than the 0% used by Domenech *et al.* (2010) in their case study of pasteurised milk. The shelf life of products is set based on the risk of spoilage and growth of pathogenic microorganisms. Increased risks will lead to shorter shelf lives. The fact that these products, which are given a shorter shelf life (e.g. 7-10 days) because there is an increased risk, have a higher probability of being consumed after their 'use by' date will again increase the risk of these products. In the

case of REPFEDs this is especially the case for products that are susceptible to recontamination with *L. monocytogenes* (Chapter 2).

The relatively short storage times in a consumer refrigerator will positively influence the safety of REPFEDs. Extended storage times, combined with temperature abuse in consumer refrigerators are important causes of elevated numbers of pathogenic microorganisms in REPFEDs (Carlin *et al.*, 2000b; Nissen *et al.*, 2002). The temperature in a consumer fridge in France can be as high as 13.8 °C and the 75 percentile of fridge temperature in Belgium is 8°C (De Vriese *et al.*, 2005; Derens *et al.*, 2004). At these temperatures, growth of psychrotrophic pathogenic microorganisms like *L. monocytogenes*, *B. cereus* and non-proteolytic *C. botulinum* is likely to occur.

To assess the exposure to psychrotrophic pathogenic microorganisms it is vital to include both time and temperature. While the design of this study does not allow inferring a correlation between storage time and temperature several studies have come to contrasting conclusions about this issue. Domenech *et al.* (2012) assumed, based on expert opinion, that there is an inverse correlation between storage time and temperature for the storage of smoked fish. Garrido *et al.* (2010) reached a similar conclusion for smoked fish and sliced cooked ham, based on a small-scale survey (n=33). The argument in both studies is that long-term storage at elevated temperatures will lead to a sensorial unacceptable product. In contrast Pouillot *et al.* (2010) found only limited support for a correlation between storage-time and temperature.

6.3.8 Considerations about the mathematical method used

The current method is a straightforward method to determine the time a product spends in a consumer fridge. Both the TTC calculation and the exposure assessment in chapter 7 used Monte Carlo simulations, to be able to later use the TTC in the exposure assessment. Monte Carlo simulations is a method of calculation used when deterministic calculation is infeasible, for example when multiplying a (large number of) probability distributions. In Monte Carlo the product is calculated by assigning a random value to each distribution, calculating the result and repeating this process many times over (100 - 1000,000 times depending on the complexity). Of course this random value is not really random, but depends on the probability distribution it represents. For example: if the probability distribution represents a fair dice being thrown, then during Monte Carlo simulations this variable can be any discrete number from 1 to 6 and each with equal probability. Another example: if the probability distribution represents two fair dice being thrown, then during Monte Carlo simulations this variable can be any discrete number from 2 to 12, but the random number will more likely be 7 (6/36) than 2 (1/36). Applying the same technique allowed us

Table 6.6: Cumulative percentage of products consumed as a function of the shelf life duration and the storage time in consumer refrigerator and the shelf life. Percentages in bold are the percentage of products that is eaten before the 'use by' date.

Storage-time (days)	Shelf life (days)			
	7	14	28	35
0	19.8%	19.8%	19.8%	19.8%
1	36.3%	36.3%	36.3%	36.3%
2	52.9%	52.9%	52.9%	52.9%
3	69.3%	69.3%	69.3%	69.3%
4	75.5%	75.5%	75.5%	75.5%
5	81.6%	81.6%	81.6%	81.6%
6	87.6%	87.6%	87.6%	87.6%
7	**93.7%**	93.7%	93.7%	93.7%
14	95.6%	**99.2%**	99.2%	99.2%
28	95.7%	99.4%	**99.5%**	99.4%
35	95.7%	99.4%	99.6%	**99.6%**
Discarded	4.3%	0.6%	0.4%	0.3%
% Consumed after 'use by' date	2.1%	0.2%	0.1%	0.1%

to directly implement the TTC-distribution in the exposure assessment without loosing flexibility (e.g. changing shelf life duration). It should be noted that the current TTC-distribution is only a point-estimate, i.e. there is no measure of uncertainty on the distribution. For example, when a consumer indicated that he bought only one REPFED per year, this was considered to be an actual "1" and not an estimated value, based on our sample. One improvement of the TTC would be to add uncertainty on the consumers' answers, for example by stating that "1 per year" is actually a probability distribution ranging from 0.5 to 3 per year (Pouillot *et al.*, 2010).

More sophisticated mathematical methods such as Bayesian inference or frequentist methods using interval-censored data might provide more accurate estimates of the TTC. However, the aim of the current study was not to compare different mathematical methods or modelling approaches, but rather to illustrate the fact that a large portion of REPFEDs is consumed shortly after purchase, and that this fact is likely to have a considerable impact on the exposure and risk associated with these products.

6.3.9 Considerations about the use of a questionnaire

The results presented above are based on the responses given by the participants and this involves a form of self-assessment by the consumer. The question remains whether the consumer is a good and unbiased "self-assessor". For example: Petty *et al.* (2013) reported that consumers were good at assessing their self reported eating rate and matched the eating rate measured under lab conditions, but did not match the free-living eating rate. Similarly it can be argued that the formulation of the answers could cause consumer bias (e.g. question 5: "strict" and "limited").

With hindsight some improvements could have been made to the questionnaire. The options in question 2 ("do you have an idea of the shelf life?") were fairly broad. More narrow options would have made this specific assessment more precise. Additionally, a control question could have been included to check the amount of REPFEDs that was discarded (e.g. "Did you discard a REPFED because if has passed the 'use by' date in the last year?"). However, despite its obvious limitations, a questionnaire is still an adequate tool to measure consumer behaviour.

6.4 Conclusion

The results of the survey show that a large segment of the population purchases REPFEDs. It also brings to light that there are significant differences in the frequency of purchase and in the storage times of these products. The combination of these data led to the development of a time-to-consumption distribution. The combination of TTC with the temperature profile in the consumer refrigerator will allow a more accurate prediction of microbial growth during consumer storage. The TTC distribution, created based on the survey, will be incorporated in the quantitative microbiological exposure assessment for *B. cereus* in REPFEDs (Chapter 7). Scenario analysis (Chapter 8) should allow quantification of the responsibility of the producer and the consumer in the microbial safety of REPFEDs. However, it must be noted that the current distribution is only based on Belgian consumers and should not be used for different (food)-cultures without due care.

Chapter 7

A Quantitative Microbiological Exposure Assessment for *Bacillus cereus* in in-pack-pasteurised REPFEDs: Part 1 - model development

Redrafted from:

Daelman, J., Membré, J. M., Jacxsens, L., Vermeulen, A., Devlieghere, F. & Uyttendaele, M. (2013e). A Quantitative Microbiological Exposure Assessment model for *Bacillus cereus* in REPFEDs. *International Journal of Food Microbiology*, 166(23):433–449

Summary

In this chapter a Quantitative Microbiological Exposure Assessment (QMEA) of psychrotrophic *B. cereus* in REPFEDs is presented. The goal is to quantify (i) the prevalence and concentration of *B. cereus* during production and distribution; (ii) the number of packages with potential toxin formation and (iii) the impact of different processing steps and consumer behaviour on the exposure to *B. cereus* from REPFEDs. The QMEA comprises the entire production and distribution process, from raw materials over pasteurisation and up to the moment it is consumed or discarded. To model this process the modular process risk model (MPRM) was used (Nauta, 2002, 2008). The product life was divided into nine modules, each module corresponding to a basic process: (1) raw material contamination, (2) cross contamination during handling, (3) inactivation during preparation, (4) growth during intermediate storage, (5) partitioning of batches in portions, (6) mixing portions to create the product, (7) recontamination during assembly and packaging, (8) inactivation during pasteurisation and (9) growth during shelf life. Each of the modules was built using a combination of newly gathered and literature data, predictive models and expert opinions. Units (batch/portion/package) with a *B. cereus* concentration of 10^5 CFU/g or more were considered 'risky' units. Results show that the main drivers of variability and uncertainty are consumer behaviour, strain variability and modelling error. The prevalence of *B. cereus* in the final products is estimated at 48.6% (±0.01%) and the number of packs with too high *B. cereus* counts at the moment of consumption is estimated at 4750 packs per million (0.48%). Four key points were identified (i) raw material contamination, (ii) recontamination during packaging, (iii) reduction during pasteurisation and cooking and (iv) cold storage at retail and consumer level.

7.1 Introduction

The microbial safety of REPFEDs is usually assured by a combination of thermal treatment (pasteurisation and cooking), packaging, cold storage and product formulation (pH, a_w). These measures are implemented by maintaining a comprehensive food safety management system (GMP/GHP, HACCP etc.). *B. cereus* is a pathogen of concern in REPFEDs because of its capabilities to grow at low temperature and to survive regular pasteurisation treatments. Given the detrimental effect of pasteurisation on product quality (texture, taste, nutrient content), producers are looking for possibilities to reduce the heat treatment without increasing the microbial risk for the consumer. To assess the current situation with respect to food safety, a quantitative microbiological exposure assessment (QMEA) for *B. cereus* in REPFEDs was developed. Risk assessment comprises four parts: (i) hazard identification, (ii) hazard characterisation (dose-

response relation), (iii) exposure assessment and (iv) risk characterisation (Codex Alimentarius Commission, 2007). Both exposure and dose-response relation are needed to assess the risk. However, in the case of *B. cereus* no reliable dose-response relation is available, because there is no straightforward link between illness and concentration of *B. cereus*. Therefore an exposure assessment is performed instead of a risk assessment.

The probability of developing food borne illness upon consumption of a given concentration of *B. cereus* depends on the type of strain (psychrotrophic/mesophilic), the physiological state of the microorganisms (cell/spore), the food product and the health of the consumer (Ceuppens *et al.*, 2011). The two food-intoxications caused by *B. cereus* (emetic and diarrhoeal) have very dissimilar causes. The emetic syndrome is caused by the ingestion of a preformed heat-stable emetic toxin (cereulide), while the diarrhoeal syndrome is caused by the ingestion of large amounts of *B. cereus* spores (Ceuppens *et al.*, 2011). The fact that only a limited number of *B. cereus* cells are able to survive gastric passage needed to initiate diarrhoeal symptoms (Ceuppens *et al.*, 2012), can be one of the reasons why Langeveld *et al.* (1996) did not see any significant effect of high *B. cereus* cell concentrations (10^8 CFU/ml) in their human volunteer study.

Nevertheless, it is generally accepted that *B. cereus* concentrations of more than 10^5 CFU/g (spores or cells) are unacceptable (Langeveld *et al.*, 1996). In part, because reviews of outbreaks and animal and human volunteer studies indicated that 10^5 CFU/g can cause food poisoning and, in part, because such elevated *B. cereus* counts are inconsistent with the principles of good manufacturing and hygiene practices (GMP/GHP) (Notermans *et al.*, 1997; Lund *et al.*, 2000). In this study, it was therefore assumed that any product containing more than 10^5 CFU/g is a risk to human health. However, a distinction was made between cells and spores. If the product contains 10^5 spores/g at any moment during production (before pasteurisation), and if that concentration is reduced to less than 10^5 spores/g due to pasteurisation, then there is no risk (given adequate storage temperatures). However, if this concentration of 10^5 spores/g is present in the final product (i.e. during the shelf life), there is a risk. Spores are able to pass the stomach more easily than vegetative cells; subsequently they can germinate, colonise the intestine and produce enterotoxins. For cells the situation is different, if a product contains 10^5 cells/g during any stage of production or shelf life, then production of the emetic toxin is possible. And since this toxin is heat stable it will still be present after pasteurisation (Rajkovic *et al.*, 2008). In short: 10^5 spores/g is not considered a risk during production (before the product is on the market), but it is considered a risk in the final product (on the market), 10^5 vegetative cells/g is considered a risk at any stage (production or distribution) because of the possibility of emetic toxin production.

Several previous exposure assessments have been done for *B. cereus*. Most notable is the Modu-

lar Process Risk Model (MPRM) by Nauta (2001, 2002, 2008). In this exposure assessment the prevalence and concentration of several *B. cereus* strains is modelled during the production and shelf life of broccoli puree. The exposure assessment compares several strains (psychrotrophic and mesophilic) as well as several distributions for the temperature in consumer fridges. Afchain *et al.* (2008) used the same basic model but compared different genetic groups of *B. cereus*. On the other hand, Malakar *et al.* (2004) performed an exposure assessment of *B. cereus* in pasteurised vegetable products using a Bayesian belief network, but did not include the distribution and consumer storage of the products.

The main difference between these assessments and the one presented in this chapter is that this exposure assessment is not designed for a specific product, but for a large variety of REPFEDs. It has been built using data and information collected in five different food companies, each producing a large variety of cooked chilled foods such as lasagne, ratatouille, spinach mash, spaghetti, etc. The exposure assessment presented in this chapter has therefore been elaborated to include a range of pH-values, a_w-values and various heat treatment regimes. Finally, an important difference with the previous work on *B. cereus* in REPFEDs, is that the injury to spores caused by the heat-treatment has been included using a *B. cereus* lag model, specifically developed for this assessment (Chapter 5).

The basic logic behind the exposure assessment is the conceptual equation (Eq. 7.1) developed by the ICMSF (2002). In this equation H_0 is the initial contamination, $\sum$R the sum of all inactivation steps, $\sum$I the sum of all growth and recontamination steps and FSO the Food Safety Objective.

$$H_0 - \sum R + \sum I \leq FSO \tag{7.1}$$

To make a clearer distinction between growth and recontamination the equation can be expanded (Eq. 7.2) with $\sum$G the sum of all growth processes and $\sum$C the sum of all recontamination processes (Zwietering, 2005). Throughout this study these notations will be used to indicate the basic process in each of the modules.

$$H_0 - \sum R + \sum G + \sum C \leq FSO \tag{7.2}$$

However, as pointed out by Havelaar *et al.* (2004), this equation is overly simplistic. It gives the impression that the calculation of the risk is just a matter of adding and subtracting. In reality this formula cannot be solved for a probabilistic model (e.g. what is the maximum H_0 given the FSO), because it is not possible to decompose the probability distributions.

The purpose of the exposure assessment presented in this study was to (i) determine the prevalence and concentration of *B. cereus* during the different steps of production and distribution (shelf life) of in-pack-pasteurised REPFEDs; (ii) quantify the number of packages with potential toxin presence and (iii) determine the impact of different processing steps and consumer behaviour on the exposure to *B. cereus* from REPFEDs.

7.2 Methodology

7.2.1 Overview of the production process

The production process of REPFEDs consists of six steps (Table 7.1 and Figure 7.1). To improve the comprehensibility of this section, the industrial production of a three-component meal will be used as an example: a meal that contains three intermediary products (IP) (e.g. a vegetable/potato component, a meat component and a sauce).

First the necessary raw materials are taken from the stock, unpacked, weighed and if necessary cleaned, cut, minced, etc. For the three-component meal this means (among many other things) that frozen products are thawed, vegetables are cut and that milk powder and herbs are weighed. This step is similar to the '*mise en place*' practice in restaurant kitchens.

Second, the different components of the REPFED are prepared. E.g. minced meat is baked, sauces are homogenised and bonded. This preparation is done in the same way as in home cooking, but on a larger scale (i.e. batches of several hundred kilogram) and this step is also the first thermal inactivation process. However, the goal of this process is not necessarily to eliminate microorganisms, but to create a certain texture, thickness or sensorial property of a component. In the three-component meal this means making sure that the vegetables are almost completely cooked and that the sauce has the desired texture.

The third step is intermediate storage. Some batches of intermediate products will have to wait for a certain time before assembly, packaging and pasteurisation. This time can range from a couple of minutes to a full day.

The fourth step is assembly and packaging. In this process large batches are first partitioned into smaller portions and then assembled (i.e. from each of the three components a small portion (50-150g) is taken and then put in a package with the other components, after which the package is sealed). For the three-component meal this means putting the three components in the tray and sealing the tray.

In the fifth step, the product is pasteurised. This final heat treatment is designed to inactivate microorganisms and the time-temperature combination of the pasteurisation process is typically a critical point in the REPFED production process.

The sixth and final step is the product shelf life. During this time, the product is stored at different locations until it is either consumed or discarded. Most of the REPFEDs are designed to be reheated by the consumer, either in the microwave or in the oven. However, this reheating is mainly for sensorial reasons. Moreover, the impact on bacterial spores is uncertain due to variability in microwave performance and consumer behaviour (Chapter 2 and 6). Therefore, the reheating step is not included in the current exposure assessment.

Table 7.1: Processing steps and their corresponding basic processes in the exposure assessment model. (-) decrease, (+) increase, (=) no change over this process.

Step	Module		Basic process	Effect on		
	N°	Code		Prevalence	N_{tot}^{a}	Unit size
1- Raw material contamination and cross contamination during '*mise en place*'	1	H_0	-			
	2	C_h	Recontamination	+	+	=
2- Thermal preparation (Cooking / baking)	3	R_c	Inactivation	-	-	=
3 - Intermediate storage	4	G_i	Growth	=	+	=
4 - Assembly and packaging of the product	5	P_a	Partitioning	-	=	-[b]
	6	M_i	Mixing	+	=	+[c]
	7	C_a	Recontamination	+	+	=
5 - Pasteurisation	8	R_p	Inactivation	-	-	=
6 - Storage in the cold chain during shelf life	9	G_s	Growth	=	+	=

[a] N_{tot}: total number of bacteria present in the unit

[b] Change from batch (100-1000kg) to portion (25-150g)

[c] Change from portion (25-150g) to package (300-1500g)

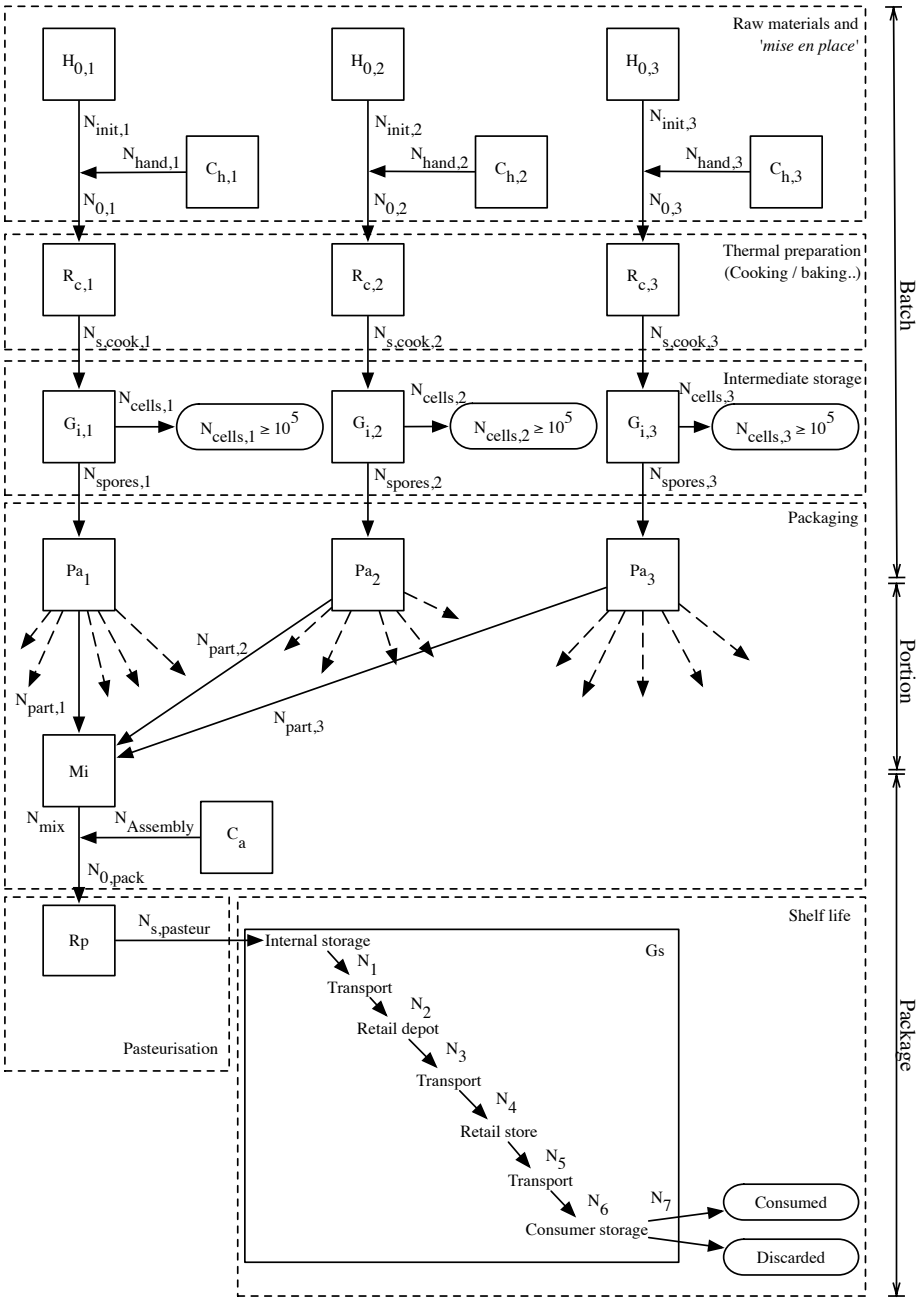


Figure 7.1: Flowchart of the modular process risk model for the production of in pack pasteurised REPFEDs. Bracketed squares delimit the six different steps in the production process. Each solid lined square marks a module - H_0: Raw material contamination; C_h: contamination during handling; R_c: reduction during preparation; G_i: growth during intermediate storage; P_a: partitioning of the batch in portions; M_i: combining the portions into a product; C_a: contamination during assembly and packaging; R_p: reduction during pasteurisation; G_s: growth during shelf life. Ovals mark endpoints that are evaluated in the exposure assessment. The subscript numbers in the first five modules (H_0 to P_a) represent the intermediate product (j:1→3). Dotted arrows are the portions that are not included in the model. The axis on the right marks the different unit sizes.

7.2.2 Set up of the Modular Process Risk Model (MPRM)

As a framework for the QMEA the modular process risk model (MPRM) presented by Nauta (2002) was used. This approach is based on the idea that any food pathway can be broken up into processing steps and that each of these steps corresponds to one of six basic processes: two microbiological processes (growth and inactivation) and four product handling processes (mixing, partitioning, removal and cross contamination).

The six production steps (from section 7.2.1) were translated into nine modules in the MPRM (Table 7.1 and Figure 7.1). The first step, the '*mise en place*', consists of two modules: first the initial contamination on the raw materials (H_0) and second the cross contamination during handling of the raw materials (C_h). The second step, cooking, corresponds to an inactivation module (R_c) and the third step, intermediate storage, is a growth module (G_i). The assembly and packaging process consists of three modules: partitioning (P_a), mixing (M_i) and recontamination (C_a). The pasteurisation process corresponds to a thermal inactivation (R_p) and the final storage during shelf life in the cold chain is again a growth module (G_s). Each of the modules is discussed in detail in the following section. Throughout the model the index j ($1 \rightarrow 3$) refers to the intermediate product (i.e. REPFED component). For each of the modules a detailed table with parameters, distributions and model equations is provided (Tables 7.2 - 7.11 on pages 173-189).

The MPRM was developed in cooperation with four REPFED producing companies. Each company provided its best estimates for the different parameters of their production process (e.g. waiting time, storage temperature). This information was combined to create a representative image for the four companies and is referred to as '*company info*'. The authors provided additional expert opinions (e.g. transport time by consumers) and these are referred to as '*expert opinion*'.

7.2.2.1 General model assumptions

As for any exposure assessment, some knowledge gaps remain for which an assumption had to be made to allow the completion of a quantitative model. The six assumptions with a model-wide impact are discussed below, other assumptions are discussed in the respective modules.

Strain diversity: *B. cereus* strains can be psychrotrophic or mesophilic strains. While the mesophilic strains are usually more heat resistant, they are unable to grow at normal refrigeration temperatures ($\leq$10°C) (Carlin *et al.*, 2006). However, the mesophilic strains may also pose a problem, either during waiting times in the production process, if the temperature is sufficiently high (>10/celsius), or in case of considerable temperature abuse at retail or consumer level. The

model used the data reported by Samapundo *et al.* (2011b), indicating that 2.6% of the *B. cereus* group spp. strains (isolated from REPFEDs and REPFED ingredients) was able to grow at 7°C, 6.2% at 8°C, 49.7% at 9°C and 87.9% at 10°C (n=380). Since multiple strains can be present on one product, it was assumed that the final population of *B. cereus* after pasteurisation followed this distribution.

Toxin forming potential. Not all strains have the necessary genes to produce the emetic or the diarrhoeal toxins. And even if they have them, these genes are not always expressed due to bacterial (growth stage, strain) or environmental conditions (temperature, atmosphere, pH, food consistency etc.) (Ceuppens *et al.*, 2011; Samapundo *et al.*, 2011b). The assumption is made that all the *B. cereus* strains present in the products have both the ability to produce emetic and diarrhoeal toxins. This can be considered a worst-case assumption. Especially given that only a small percentage of *B. cereus* strains is considered to have the ability to produce the emetic toxin (Samapundo *et al.*, 2011b; Altayar & Sutherland, 2006; Carlin *et al.*, 2006). However, it is unsure which strains will be present during the production, which strains will grow first, etc. The effect of this parameter was therefor tested (i.e. % of strains which produce emetic toxins) in a separate scenario in chapter 8.

Spores vs. cells: Spore forming bacteria have two physiological states (i.e. vegetative cells and spores). Consequently, *B. cereus* can be present in the raw materials or in the environment in both states. However, the distributions for the contamination of raw materials and the production environment were based on the data gathered in chapter 2, which does not differentiate between cells or spores. It is therefore assumed that all *B. cereus* entering the model are spores, be it in the raw materials or through recontamination . Again, this is a worst-case assumption, because only spores will survive the thermal processing.

Sporulation and germination: The rate at which bacteria transfer from one state to the other (i.e. from spores to cells and back) is variable. Although some models for spore germination are available (Collado *et al.*, 2006; Sinigaglia *et al.*, 2002), none of these was deemed suitable for application in the QMEA. Therefore two assumptions were made:

1. Sporulation (transformation from cells to spores) only occurs at the end of the growth process, when bacterial counts have already exceeded the acceptable limit (Nauta, 2001). It was therefore considered not to take place during the production process and was thus excluded from the model.

2. Germination (transformation from spores to cells) is important because cells that germinate during intermediate storage are inactivated during subsequent thermal processing. How-

ever, a certain portion of the spores will remain dormant, even at optimal conditions and can survive the pasteurisation process (EFSA, 2005a). The assumption is made that germination occurs immediately after the lag time (i.e. no germination lag) and that the percentage of spores that does not germinate was minimum 0%, most likely 0.01% and maximum 30% (Nauta, 2001).

Spoilage: Spoilage is a relevant factor when considering the storage of products, especially under temperature abuse. However, the range of REPFEDs is considered too diverse to make an accurate estimation of the conditions that lead to noticeable spoilage. It is therefore assumed that no spoilage takes place. This is again a worst-case assumption.

Minimal heat treatment: As a precaution against unrealistic predictions, a minimal application limit is given for the heat treatment. The model is set to require a minimum heat treatment of 70°C for 2 minutes (or 72°C for 1 minute). Without this minimal heat treatment other (vegetative) pathogens may survive and pose a risk (e.g. *Listeria monocytogenes*) (Farber & Peterkin, 1991).

7.2.2.2 Raw material contamination (H_0 - module 1)

To cover the wide range of REPFEDs available on the market, raw materials were divided into six groups:

1. Starch components (e.g. flour, potatoes, rice, pasta, starches)
2. Dry herbs, spices and powders (e.g. pepper, dried basil, dried oregano, milk powder)
3. Meat, fish and dairy products (e.g. veal, codfish, cream, butter)
4. Fruit and vegetable products (e.g. apples, tomatoes)
5. Ambient stable products (e.g. canned products, olive oil, wine)
6. Water

For the first five groups a continuous distribution of the *B. cereus* contamination was developed using a Bayesian model (Chapter 3) with data gathered in a previous study (Chapter 2). To prevent the model from simulating excessively high *B. cereus* counts in the raw materials, the distributions were truncated on the right side (Table 7.2, p.173). Based on expert opinion the truncation was set at a 10^6 CFU/g. This concentration is the sum of the maximum tolerable *B.*

cereus concentration in raw materials (10^4 CFU/g, Uyttendaele *et al.* (2010)) and a measure for uncertainty and variability (10^2 CFU/g, expert opinion).

This module allows entering a crude recipe; i.e. a percentage (w/w) for each of the six groups. Based on these percentages and on the respective contamination of each category, the initial contamination ($H_{0,j}$) of each of the three intermediate products is calculated (Table 7.2, p.173).

7.2.2.3 Cross contamination during handling of raw materials (C_h - module 2)

The cross contamination during the handling of raw materials (e.g. portioning, weighing, mixing, transportation) prior to the thermal preparation was split into two parts depending on the source of the contamination: direct and indirect contamination (Table 7.3, p. 175). Direct contamination is contamination that originates from materials that make contact with the food product (e.g. spoon, mixer, hands, gloves, etc.). Indirect contamination originates from materials that do not make contact with the product (e.g. walls, ceiling, non contact parts of machines, etc.). During a previous study samples of the environment during handling were taken (sampling location 8-11 in Chapter 2). Using the R-package *fitdistrplus* (Delignette-Muller *et al.*, 2010) and these samples of the handling environment, a distribution was created for each of the two types of contamination sources (expressed as log CFU/25cm^2). Starting from these distributions the total cross contamination was calculated using a different model for each of the two types of contamination.

For direct contamination ($C_{\text{Direct,hand,j}}$) it was assumed that during each contact a *B. cereus* spore had a certain probability of being transferred from the source to the product. If each of the contacts occurred with the same object (e.g. a spoon), then the contamination on this object would decrease with each contact. However, it is unlikely that the product would only make contact with one object. It was therefore assumed that the contamination on the source remained the same, irrespective of the number of transfers or the transfer rate; i.e. that the source was very large in comparison with the amount of spores transferred. For the transfer rate a uniform distribution was used, based on the transfer rates proposed by Luber *et al.* (2006) for the transfer of *Campylobacter* spp. from hands or kitchen utensils to ready-to-eat foods. Rather than just using the transfer rate as a percentage (e.g. 30% is transferred on each contact) a binomial distribution ($N \cdot Nr, p$) was used (expert opinion) with N the number of *B. cereus* spores present on the source, Nr the number of manipulations or contacts and p the probability of transfer (i.e. the transfer rate).

For indirect contamination ($C_{\text{Indirect,Hand,j}}$) the model described by den Aantrekker *et al.* (2003)

was used. For the two transfer-rate coefficients (k_{i} and k_{p}) the values for transfer from hands via air to the product were used. The values for hands were preferred to those for air and biofilms because during the sampling of the original data, used for this distribution, no biofilm- or air-samples were taken (chapter 2).

After calculation of both values (direct and indirect) for each intermediate product, the combined cross contamination ($C_{\mathrm{h,j}}$) value was added to the respective initial contamination ($H_{0,\mathrm{j}}$). The sum of initial contamination and cross contamination yielded the number of spores present in the intermediate product prior the first heat treatment ($N_{0,\mathrm{j}}$).

7.2.2.4 Inactivation during preparation of intermediate products ($\mathbf{R_c}$ - module 3)

Once the intermediate products are assembled, they are thermally prepared. The goal of the preparation step is not eliminating microorganisms, but to achieve technological/sensorial goals (e.g. binding of a sauce, browning of meat). While the heat treatment may not be designed to inactivate microorganisms, it will inactivate a portion of the microorganisms present (Table 7.4, 178). To model the thermal inactivation as a function of time and temperature during preparation, the commonly used log linear D-/z- approach was used (section 1.3.3.1, p.19), with the values proposed by van Asselt & Zwietering (2006) for *B. cereus*. To account for strain variability, a normal distribution was used for the logarithm of the D-values (log (D_{ref})). The effect of a_{w}, pH and other variables (shoulders, tails) was not taken into account, based on the finding by van Asselt & Zwietering (2006) that these effects are negligible in comparison to the inter-strain variability.

The inactivation was modelled using a Poisson ($N \cdot p$) distribution, with N the number of spores entering and p ($= 10^{(-t/D)}$) the probability a spore survives the heat treatment. The Poisson distribution was used as approximation for a Binomial (N,p) distribution (Nauta, 2001) because the @Risk software has calculation issues with the Binomial distribution for large values of N and small values of p.

7.2.2.5 Growth during intermediate storage of intermediate products ($\mathbf{G_i}$ - module 4)

After preparation, there is usually some waiting time before the products are packed and pasteurised (Table 7.5, 179). Depending on the production process, the product is stored hot (>55°C) or cold (≤4°C) during this time. However, even if the product is cooled, it can still remain warm (20-55°C) during this storage, because of the large volume of the batches. During this storage

time spores that are present can germinate and grow. The rate of germination and growth depends on the heat treatment during preparation, the storage temperature and the intrinsic product properties.

The module for growth during storage uses two distinct models. The first model is a gamma-type lag model for heat-treated psychrotrophic *B. cereus* spores under cold storage (Chapter 5). The second is a gamma-type model for the growth rate (Augustin *et al.*, 2005). The predicted growth rate is used as input for the primary growth model by Rosso *et al.* (1996). The cardinal values for the effect of temperature in the model for growth rate ($T_{G,min}$, $T_{G,opt}$, $T_{G,max}$) were obtained from Membré *et al.* (2005), the cardinal values for pH and a_w as well as μ_{opt} were deduced from predictions on rice products provided by the Sym'previus software (http://www.symprevius.net). Although the combination of two different models for lag and growth rate is not optimal, it is currently the only available option given the complex nature of the problem (spore germination, cell lag, heat treatment, cold storage) and the lack of a predictive model for both lag time and growth rate.

Spores that germinate during intermediate storage, become cells, lose their heat resistance and are subsequently eliminated during pasteurisation. This reduces the exposure further down stream. However, if the concentration of cells exceeds 10^5 CFU/g, there is a risk of emetic toxin formation. To take this double effect of storage time in account, this module calculates the lag time. If the lag time is shorter than the storage time, spores can germinate (Figure 7.2). For the spores that germinate, the growth rate is calculated and growth is modelled during the remaining time (storage - lag time). If the concentration of *B. cereus* cells does not exceed 10^5 CFU/g, these cells are not included in the rest of the model, because they are unable to survive the subsequent pasteurisation treatment. However, some spores will not germinate and are able to survive the heat treatment, these spores remain present and were also considered in the rest of the model. If the concentration of cells exceeds 10^5 CFU/g, the batch and the products made from this batch are labelled as unacceptable. As mentioned in section 7.2.2.1, sporulation is not considered because the time frame in which the production takes place is too short (Nauta, 2001). In short, the output of this module is double. The first output is the concentration of cells $N_{cells,j}$, which is only compared to the threshold ($\geq 10^5$ CFU/g = batch unacceptable), but not used for the rest of the model calculations. The second output is the concentration of spores ($N_{spores,j}$), which is used as input for the next module.

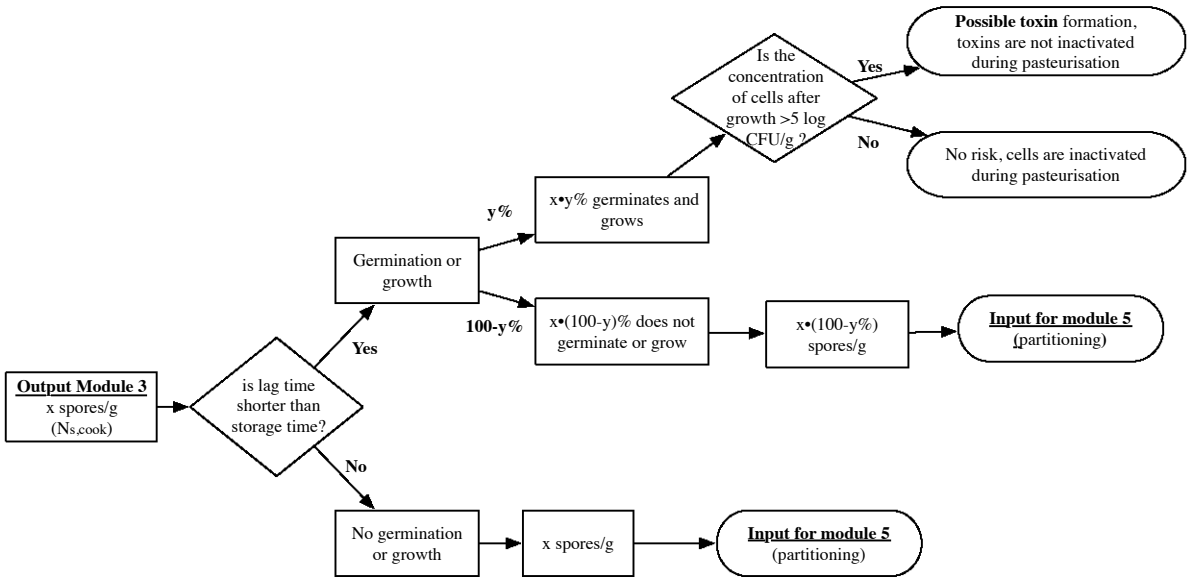


Figure 7.2: Flowchart illustrating the effect of germination and growth during intermediate storage (G_i, module 4) on the concentration of spores and cells and on the possible formation of toxins.

7.2.2.6 Partitioning (P_a - module 5) and mixing (M_i - module 6)

Partitioning and mixing are the first two modules in the assembly and packaging step (Table 7.6 and 7.7, p. 183). In these modules, a portion (e.g. 150g) is taken from the batch (e.g. 400kg) and combined with the portions from the other intermediate products to form the final product. In the case of a three-component meal, this means putting the components in the tray. During the partitioning of a large volume (i.e. the batch) in a smaller volume (i.e. the portion), there is always a probability that a certain portion contains no *B. cereus* ($P(0)$). To model the partitioning, two assumptions were made. First, the model assumes a homogeneous distribution of the spores throughout the batch. This is probably not the case in reality, since spores that originate from the same contamination event (e.g. a spot contamination from a water droplet) will be in a specific part of the batch and not homogeneously spread over the entire volume. In reality the probability that a portion is contaminated depends on the spatial distribution of the spores. But there were no data available about this spatial distribution.

Secondly, it is assumed that there are no losses: i.e. that the sum of spore counts ($\sum N_{\text{part,j}}$) in all portions (x_j) equals the initial number of spores present in the batch ($N_{\text{spores,j}}$) (Eq. 7.3) (Nauta,

2005).

$$N_{spores,j} = \sum_{i=1}^{x_j} N_{part,j} \tag{7.3}$$

To model the partitioning the approach proposed by Nauta (2005) for the contamination of one of n equally size smaller units (in this case portions) from a homogeneously contaminated batch was applied. Indeed, as only one smaller unit (a portion) per simulation was sampled, it was not necessary to consider the dependence between smaller units (portions) created from the same larger unit (batch). The distribution of the contamination is Binomial($N_{spores,j}$, $1/x_j$), which is approximated with a Poisson($N_{spores,j} \cdot 1/x_j$). The number of cells in a smaller unit (a portion) is determined as a sample from this Poisson distribution.

Modelling the mixing was straightforward since the number of cells in each portion was known (Table 7.7, p. 183). The large unit (i.e. the final product) contained the sum of all cells present in the smaller units (i.e. portions) (Nauta, 2005).

7.2.2.7 Recontamination during assembly of the product (C_a - module 7)

This module is the third and last module representing the assembly and packaging process. As described above, the batches are divided in portions and then combined to form a product. During this process, and during the subsequent actual packaging (sealing the tray) recontamination is possible. The methodology is identical to that in module 2 (recontamination during handling, section 7.2.2.3) and is given in Table 7.8 (p. 184). The distribution of *B. cereus* spore concentrations on the contact and non-contact materials was also modelled using the R-package *fitdistrplus* (Delignette-Muller *et al.*, 2010) and the samples of the packaging process environment described in Chapter 2 (sampling locations 13-14). For each of the two types (contact / non-contact) a distribution was created (expressed in log CFU/25cm^2).

After calculation of both values (direct and indirect), the combined recontamination (C_a) was added to the initial contamination of package after mixing (N_{mix}). The sum of the spores present after mixing (module 6) and the recontamination (module 7) yielded the number of spores present prior to the pasteurisation of the final product ($N_{0,pack}$).

7.2.2.8 Inactivation during pasteurisation of the assembled products (R_p - module 8)

Once the products are packed in trays or bags, a pasteurisation process is applied. Contrary to the thermal process in module 3, the goal of this process is microbial inactivation. For inactivation

during pasteurisation the methodology described in section 7.2.2.4 was used, it is nearly identical and given in Table 7.9 (p. 187).

7.2.2.9 Growth during storage in the cold chain (G_s - module 9)

After pasteurisation the product is chilled to 4°C as fast as possible (EFSA, 2005a). Once the desired temperature has been reached, the product shelf life begins. In the model the shelf life is divided into seven stages, the time and temperature distribution for each stage are given in Table 7.10 (p. 188).

1. Internal storage at producer
2. Transport from producer to retail depot
3. Storage at retail depot
4. Transport from retail depot to retail store
5. Storage at retail store
6. Transport from retail store to consumer
7. Storage in consumer fridge

To describe the behaviour (lag and growth rate) of *B. cereus* under dynamic conditions of temperature at the different stages, the '*work to be done*' approach was used. This approach assumes that the product of lag time and growth rate is constant, and that this product is the amount of work a bacterial cell has to do before it can grow (Koutsoumanis, 2001; Baranyi & Roberts, 1994; Panagou & Nychas, 2008). This meant that for each stage the lag time (in hours) and growth rate (1/h) were calculated (using the same predictive models as in section 7.2.2.5) and the product of these two (W_n) was considered as '*the work to be done*'. At each stage a certain amount of work was done (time x growth rate), which was subsequently subtracted from '*the work to be done*' in the next stage. Once the amount of '*work to be done*' reached zero, the lag phase had ended and growth started. Although this method is an approximation, it remains a useful method that has been extensively used in literature (Robinson *et al.*, 1998; Amezquita *et al.*, 2005; Peleg & Corradini, 2011; Baranyi & Roberts, 1994), especially given the absence of a combined model for lag and growth of heat-treated *B. cereus* spores. All equations and parameters used for the calculation of lag time, growth rates and bacterial concentrations are given in Table 7.11 (p. 189).

The time spent in a consumer refrigerator was modelled using the 'time to consumption' (TTC) distribution, developed in Chapter 6. This distribution is based on the results of a questionnaire, in which consumers were asked how long they stored REPFED they had bought. The distribution showed that ±50% was consumed within two days, ±75% in 5 days and ±99% in 14 days.

7.2.3 Model software and simulations

The model was implemented in @Risk software (Palisade Corporation, NY, USA). Each simulation consisted of 10^6 iterations using Latin Hyper Cube (LHC) sampling. This number was chosen as a compromise between the need for accuracy and available computing power. LHC sampling divides the samples based on the percentiles. For example, if a run has twenty iterations then the LHC method will take one value from each 5^{th} percentile. This implies that if the model contains one distribution, at least 20 iterations are needed to have one sample from the 95^{th} percentile (i.e. the right tail). However, if the model contains two distributions then at least 400 (20^2) are needed to have one iteration in which the value for both distributions is in the 95^{th} percentile. The QMEA contains a high number of stochastic parameters (± 50). This implies that to have one iteration in which the value for each distribution is in the 95^{th} percentile, 20^{50} iterations are needed ($1.13 \cdot 10^{65}$). Since this was not feasible, a number of trail simulations was done with 10^6 and 10^7 iterations. Simulations with 10^7 iterations took six times longer (1 hour vs. 10 minutes) than simulations with 10^6 iterations and results and model convergence were similar.

7.2.4 Sensitivity analysis

To determine which of the input distributions had the largest effect on the exposure, a global sensitivity analysis was performed. This implies that the model was run twice for each of the distribution in the model (e.g. Concentration in starch components, D-value, temperature at consumer, etc.). In the first run the distribution was fixed at its 1^{st} percentile value (e.g. very low D-value) while all other distributions were unchanged (i.e. remained variable). In the second run the distribution was fixed at its 99^{th} percentile value (e.g. very high D-value) while all other distributions remained variable. The difference in the final output (i.e. packs with more than 10^5 CFU/g) between these two simulations reflects how sensitive the model output was to this specific input distribution.

7.3 Distributions and models used in the QMEA

This section contains ten tables (7.2-7.11), each table list the parameters, distributions and model-equations used in the different modules or the time/temperature distributions used in module 9. Detailed information about the choice of these parameters and models is available in section 7.2.

Overview of the tables in this section:

Table 7.2: Parameters, distributions and models used in module 1: raw material contamination for IP= j:1→3

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
Spec	Maximum tolerated *B. cereus* concentration on raw materials as defined in company quality manual (i.e. specification to supplier)	log CFU/g	4	Uyttendaele *et al.* (2010)
Trunc	Right truncation on distributions of raw material contamination	log CFU/g	Spec + 2	Expert opinion
Starch	Contamination of starch components in stock	log CFU/g	RiskNormal(RiskNormal(-4.07, 1.16), 3.31, Risktruncate(,Trunc))	Chapter 3
Herbs	Contamination of dry herbs, spices and powders in stock	log CFU/g	RiskNormal(RiskNormal(-2.73, 0.77), 3.31, Risktruncate(,Trunc))	
Meat	Contamination of meat, fish and dairy products in stock	log CFU/g	RiskNormal(RiskNormal(-5.16, 1.19), 3.31, Risktruncate(,Trunc))	
Fruit	Contamination of fruit and vegetable products in stock	log CFU/g	RiskNormal(RiskNormal(-6.38, 1.61), 3.31, Risktruncate(,Trunc))	
Ambient	Contamination of ambient stable products in stock	log CFU/g	RiskNormal(RiskNormal(-7.14, 3.15), 3.31, Risktruncate(,Trunc))	
Water	Contamination of the water used[b]	CFU/g	0	Expert opinion
$\%_{starch,j}$	% of starch components in IP j (j: 1→3)	% [w/w]	15%	Company info[c]
$\%_{Herbs,j}$	% of dry herbs, spices and powders in IP j (j: 1→3)	% [w/w]	1%	
$\%_{meat,j}$	% of s meat, fish and dairy products in IP j (j: 1→3)	% [w/w]	24%	

Continued on next page

Table 7.2 – continued from previous page

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
$\%_{\text{fruit,j}}$	% of fruit and vegetable products in IP j (j: 1→3)	% [w/w]	15%	Company info[c]
$\%_{\text{ambient,j}}$	% of ambient stable in IP j (j: 1→3)	% [w/w]	15%	
$\%_{\text{water,j}}$	% of water in IP j (j: 1→3)	% [w/w]	30%[c]	
$M_{\text{batch,j}}$	Weight of a batch op IP j	kg	400	
$H_{\text{starch,j}}$	Contamination of starch components used	$\frac{\text{spores}}{\text{batch}}$	$=\text{Round}\left(\%_{starch,j} \cdot M_{batch,j} \cdot 1000 \cdot 10^{Starch}, 0\right)$	
$H_{\text{Herbs,j}}$	Contamination of dry herbs, spices and powders used	$\frac{\text{spores}}{\text{batch}}$	$=\text{Round}\left(\%_{herbs,j} \cdot M_{batch,j} \cdot 1000 \cdot 10^{Herbs}, 0\right)$	
$H_{\text{meat,j}}$	Contamination of meat, fish and dairy products used	$\frac{\text{spores}}{\text{batch}}$	$=\text{Round}\left(\%_{meat,j} \cdot M_{batch,j} \cdot 1000 \cdot 10^{Meat}, 0\right)$	
$H_{\text{fruit,j}}$	Contamination of fruit and vegetable products used	$\frac{\text{spores}}{\text{batch}}$	$=\text{Round}\left(\%_{fruit,j} \cdot M_{batch,j} \cdot 1000 \cdot 10^{Fruit}, 0\right)$	
$H_{\text{ambient,j}}$	Contamination of ambient stable products used	$\frac{\text{spores}}{\text{batch}}$	$=\text{Round}\left(\%_{ambient,j} \cdot M_{batch,j} \cdot 1000 \cdot 10^{Ambient}, 0\right)$	
$N_{\text{init,j}}$	Initial contamination of IP j	$\frac{\text{spores}}{\text{batch}}$	$= H_{starch,j} + H_{Herbs,j} + H_{meat,j} + H_{fruit,j} + H_{ambient,j}$	

[a] If applicable

[b] although the water is not considered to be contaminated with *B. cereus*, the percentage is asked to verify that the total amounts to 100%

[c] data provided by four REPFED producing companies.

Table 7.3: Parameters, distributions and models used in module 2: recontamination during handling of raw materials for IP= j:1→3

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
Part 1: Contamination due to direct contact from contact materials (e.g spoons, mixers,etc.)				
ContactHand	Contamination of contact materials used during the handling of the intermediate products	$\frac{\log \text{ spores}}{25cm^2}$	RiskNormal(-0.20, 1.21)	Based on data in Chapter 2
$Nr_{\text{Contacts,hand,j}}$	Number of times contact is made between the product and a contact material (e.g. spoon) during handling	-	RiskIntUniform(1,10)	Companies
T_{r}	Transferrate between contact material and hand	%	Riskuniform(2.9%, 27.5%)	Luber *et al.* (2006)
$S_{\text{contact,hand,j}}$	Average size of contact material	cm^2	100	Company info
$N_{\text{spores,contact,hand,j}}$	Number of spores on contact material	Spores	$=\text{Round}\left(\frac{10^{ContactHand} \cdot S_{Contact,Hand,j}}{25}, 0\right)$	
$C_{\text{Direct,hand,j}}$	Direct contamination during handling of IP j (1 →3)	$\frac{\text{spores}}{\text{batch}}$	$=\text{RiskBinomial}\left(N_{spores,contact,hand,j} \cdot Nr_{Contacts,hand,j}, Tr\right)$	
Part 2: Contamination due to **indirect contact** from the environment (e.g walls, machines,etc.)				
NonContactHand	Contamination on the environment (non-contact) materials during handling of the intermediate products (e.g. wall)	$\frac{\log \text{ spores}}{25cm^2}$	RiskNormal(-1.59, 1.49)	Based on data in Chapter 2
$S_{\text{environ,hand,j}}$	Total surface of walls, floors, etc in handling environment. Room of 3x3x3m	m^2	54	Expert opinion

Continued on next page

Table 7.3 – continued from previous page

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
$V_{\text{air,hand,j}}$	Volume of the room where handling takes place. Room of 3x3x3m	m^3	27	
$S_{\text{batch,j}}$	Surface of batch of IP j susceptible to recontamination	m^2	0.25	Company info
$t_{\text{hand,j}}$	Time exposed to the environment in handling zone of IP j	hr	1.5	
k_{i}	Transferrate coefficient from source (=surface) to air	1/h	RiskUniform (0.01, 10)	
k_{p}	Transferrate coefficient from air to batch	m/h	RiskUniform (0.01, 200)	den Aantrekker *et al.* (2003)
$M_{\text{batch,j}}$	Weight of a batch op IP j (same as in module 1)	kg	300	Company info
$C_{\text{source,hand,j}}$	Contamination of source (=surfaces)	Spores/m^2	$=10^{NonContactHand} \cdot \frac{1000}{25}$	
$K_{\text{i,hand,j}}$		1/h	$= \frac{S_{environ,hand,j} \cdot k_i}{V_{air,hand,j}}$	den Aantrekker *et al.* (2003)
$K_{\text{p,hand,j}}$		1/h	$= \frac{S_{batch,j} \cdot k_p}{V_{air,hand,j}}$	
$C_{\text{p,hand,j}}$	Contamination of batch of IP j per square meter	$\frac{\text{Spores}}{m^2}$	$= K_{i,hand,j} \cdot C_{source,hand,j} \cdot \left[t_{hand,j} + \frac{1}{K_{p,hand,j}} \cdot \left(exp\left(-K_{p,hand,j} \cdot t_{hand,j}\right) - 1 \right) \right]$	den Aantrekker *et al.* (2003)[b]
$C_{\text{Indirect,Hand,j}}$	Indirect contamination during handling of IP j	$\frac{\text{spores}}{\text{batch}}$	$=\text{Round}(C_{p,hand,j} \cdot S_{batch,j}, 0)$	

Continued on next page

Table 7.3 – continued from previous page

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
Part 3: Adding both types of recontamination to calculate total recontamination				
$N_{\text{hand,j}}$	Total recontamination due to handling of a batch op IP j	$\frac{\text{spores}}{\text{batch}}$	$= C_{Indirect,Hand,j} + C_{Direct,hand,j}$	

[a] If applicable

Table 7.4: Parameters, distributions and models used in module 3: inactivation during cooking of intermediate products IP= j:1→3

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
$HTT_{\text{cook,j}}$	Temperature of the thermal treatment of IP j	°C	90	Company info
$HT, t_{\text{cook,j}}$	Time of the thermal treatment of IP j	min	20	
T_{ref}	Reference temperature	°C	120	van Asselt & Zwietering (2006)
z	Thermal death constant	°C	12.8	
$\log(D_{\text{ref}})$	Distribution of D-value's for *B. cereus*	min	RiskNormal(-1.38, 0.56)	
D_{j}	D-value at HTTcook,j	min	$= 10^{D_{ref}} \cdot 10^{\left(\frac{-\left(HTT_{cook,j}-T_{ref}\right)}{z}\right)}$	Holdsworth (2004)
p_{j}	Probability that a spore survives the heat treatment applied to IP J	-	$= 10^{\left(\frac{-HT,t_{cook,j}}{D_j}\right)}$	
$N_{0,\text{j}}$	Spores present in batch of IP j before cooking	$\frac{\text{spores}}{\text{batch}}$	$= N_{init,j} + N_{hand,j}$	
$N_{\text{s,cook,j}}$	Spores surviving the cooking of IP j	$\frac{\text{spores}}{\text{batch}}$	=RiskPoisson$(N_{0,j} \cdot p_j)$	Nauta (2001) [b]

[a] If applicable

[b] Number of spores surviving the pasteurisation described by a Binomial distribution (N_0, p_{j}) (Nauta, 2001) and simplified by a Poisson distribution as N_0 is large and p_{j} small.

Table 7.5: Parameters, distributions and models used in module 4: growth during intermediate storage of the intermediate products (IP= j:1→3)

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
Part 1: Declaration of **batch properties** and **storage conditions**				
$N_{\text{s,cook,j}}$	Spores surviving the cooking of IP j	$\frac{\text{spores}}{\text{batch}}$	Output of module 3	
$M_{\text{batch,j}}$	Weight of a batch op IP j	kg	400 (= module 1)	Company info
$HTT_{\text{cook,j}}$	Temperature of the thermal treatment op IP j	°C	90 (= module 3)	
$HT, t_{\text{cook,j}}$	Time of the thermal treatment of IP j	min	20 (= module 3)	
$a_{w,\text{j}}$	a_w of IP j	-	0.990	Chapter 2_b
pH_{j}	pH of IP j	-	5.95	
$t_{\text{wait,j}}$	Waiting time between preparation and packaging for IP j	hr	RiskUniform(0,4)	Company info
StT_{j}	Storage temperature between preparation and packaging for IP j	°C	RiskPert(7,20,60)	
Part 2: Determination of **lag time** (model from chapter 5 for strain 1)				
Calculate $f_1(StT_{\text{j}})$, $f_1(pH_{\text{j}})$, $f_3(a_{w,\text{j}})$, $f_4(HTT_{\text{cook,j}})$, $f_4(HT, t_{\text{cook,j}})$ and $f_4(HT_{\text{cook,j}})$				Chapter 5 (eq. 5.4-5.9)
Calculate $\prod_i^k \gamma_i$				Chapter 5 (eq. 5.3)
ε	Error on the lag model		=RiskNormal(0, 1.36)	Chapter 5

Continued on next page

Table 7.5 – continued from previous page

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
$Lag_{\text{calc,inter,j}}$	Calculated lag time during intermediate storage after the cooking proces	days	$lag = exp\left(a_1 \cdot \prod_i^k \gamma_i - 1 + \varepsilon\right) - 1$	Chapter 5 (eq. 5.2)
$Lag_{\text{inter,j}}$	Lag time during intermediate storage corrected for model accuracy	hr	$\begin{cases} lag_{inter,j} = lag_{calc,inter,j} \cdot 24 & \text{if } lag_{calc,inter,j} \geq 1 \\ lag_{inter,j} = 0 & \text{if } lag_{calc,inter,j} < 1 \end{cases}$	
Part 3: Determine **growth rate** during intermediate storage (Augustin *et al.* (2005); Membré *et al.* (2005) and www.symprevius.net)				
μ_{opt}	Growth rate under optimal conditions	ln/h		www.symprevius.net
$\gamma(StT_{\text{j}})$	Effect of storage temperature on the growth rate	-	$\text{CM}_2(StT_j)$	
$\varphi(StT_{\text{j}})$	Interaction term for storage temperature	-	$= \left(1 - \sqrt{\gamma(StT_j)}\right)^2$	
$\gamma(a_{w,\text{j}})$	Effect of a_w on the growth rate	-	$\text{CM}_1(a_{w,j})$	Augustin *et al.* (2005) [c]
$\varphi(a_{w,\text{j}})$	Interaction term for a_w	-	$= \left(1 - \gamma(a_{w,j})\right)^2$	
$\gamma(pH_{\text{j}})$	Effect of pH on the growth rate	-	$\text{CM}_1(\text{pH}_j)$	
$\varphi(pH_{\text{j}})$	Interaction term for pH	-	$= \left(1 - \gamma(\text{pH}_j)\right)^2$	

Continued on next page

Table 7.5 – continued from previous page

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
ξ_{j}	Combined interaction term		$= \begin{cases} 1, & \psi_j \leq 0.5 \\ 2(1-\psi_j), & 0.5 < \psi_j < 1 \\ 0, & \psi_j > 1 \end{cases}$ and $\psi_j = \sum_i \frac{\varphi(i_j)}{2 \cdot \prod_{h \neq j} \left(1 - \varphi(h_j)\right)}$	
$\mu^*_{\mathrm{inter,j}}$	Growth rate	Ln/h	$= \mu_{opt} \cdot \gamma(StT_j) \cdot \gamma(\mathrm{a}_{w,j}) \cdot \gamma(\mathrm{pH}_j) \xi_j$	
SE	Error on growth rare	-	=0.05	Membré *et al.* (2005)
$\mu_{\mathrm{inter,j}}$	Growth rate of *B. cereus* in batch of IPj		$= \begin{cases} \mathrm{Risknormal}\left(\sqrt{\mu^*_{inter}}, \mathrm{SE}\right)^2, & \text{if } t_{wait,j} > lag_{inter,j} \\ 0, & \text{if } t_{wait,j} \leq lag_{inter,j} \end{cases}$	
Part 4: Determine the **concentration of cells and spores** after intermediate storage				
$\%nongerm$	Percentage of spores not germinating	-	=RiskPert(0, 0.0001, 0.3)	Nauta (2001)
$\%germ$	Percentage of spores germinating		$= \begin{cases} 0, & \text{if } t_{wait,j} < lag_{inter,j} \\ 1 - \%nongerm, & \text{if } t_{wait,j} \geq lag_{inter,j} \end{cases}$	
N_{max}	Maximum *B. cereus* concentration	log CFU/g	8.5	Expert opinion
$N_{\mathrm{max,batch,j}}$	Maximum number of*B. cereus* in a bach of IP j	CFU/batch	$= M_{batch,j} \cdot 10^{N_{max}} \cdot 1000$	
$N^*_{0,\mathrm{j}}$	Concentration of cells able to grow (ie. Germinated cells)	CFU/batch	$= N_{s,cook,j} \cdot \%germ$	

Continued on next page

Table 7.5 – continued from previous page

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
$N_{cells,j}$	Concentration of cells after intermediate storage	CFU/batch	$= \begin{cases} 0, & \text{if } N^*_{0,j} = 0 \\ Round\left(\left(\frac{N_{max,batch,j}}{1+\left[\left(\frac{N_{max,bacth,j}}{N^*_{0,j}}-1\right)\cdot exp\left(-\mu_{inter,j}\cdot\left(t_{wait,j}-lag_{inter,j}\right)\right)\right]}\right),0\right) & \text{if } N^*_{0,j} > 0 \end{cases}$	Rosso *et al.* (1996)
$N_{spores,j}$	Concentration of spores after intermediate storage	Spores/batch	$= N_{s,cook,j} - N^*_{0,j}$	

[a] If applicable

[b] a_w and pH were measured during the determination of microbiological quality

[c] $CM_n(X) = \begin{cases} 0, X \le X_{min} \\ \frac{(X-X_{max})(X-X_{min})^n}{(X_{opt}-X_{min})^{n-1}\left[(X_{opt}-X_{min})(X-X_{opt})-(X_{opt}-X_{max})((n-1)X_{opt}+X_{min}-nX)\right]} \end{cases}$

Table 7.6: Parameters, distributions and models used in module 5: partitioning of batches into portions (for IP= j:1→3)

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
$N_{\text{spores,j}}$	Concentration of spores after intermediate storage	$\frac{\text{spores}}{\text{batch}}$	Output of module 4	
$M_{\text{batch,j}}$	Weight of a batch of IPj	kg	400 (see module 1)	Company info
$M_{\text{Portion,j}}$	Weight of a portion of IPj in final product	g	150	
x_{j}	Number of portions of IP j	-	= Rounddown $\left(\frac{M_{batch,j} \cdot 1000}{M_{portion,j}}; 0\right)$	
$N_{\text{part,j}}$	Contamination of a portion of IP j after partitioning	$\frac{\text{spores}}{\text{portion}}$	= RiskPoisson $\left(N_{spores,j} \cdot \frac{1}{x_j}\right)$	Nauta (2005)

[a] If applicable

Table 7.7: Parameters, distributions and models used in module 6: Mixing of portions into products (for IP= j:1→3)

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
M_{product}	Product weight	g	= $\sum_{j=1}^{3} M_{portion,j}$ (=450g)	
N_{mix}	*B. cereus* concentration in mixed product	$\frac{\text{Spores}}{\text{package}}$	= $\sum_{j=1}^{3} N_{part,j}$	Nauta (2005)

[a] If applicable

Table 7.8: Parameters, distributions and models used in module 7: recontamination during assembly and packaging of the product

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
Part 1: Contamination due to **direct contact** from contact materials (e.g spoons, mixers,etc.)				
$ContactAssem$	Contamination of contact materials used during the assembly of the products	$\frac{\log \text{spores}}{25cm^2}$	RiskNormal(0.0842, 1.01)	Based on data in Chapter 2
$Nr_{\text{ContactsAssem}}$	Number of times contact is made between the product and a contact material (e.g. spoon) during assembly	-	RiskIntUniform(1,10)	Company info
T_{r}	Transferrate between contact material and hands	%	Riskuniform(2.9%, 27.5%)	Luber *et al.* (2006)
$S_{\text{contact,Assem}}$	Average size of contact material	cm^2	100	Company info
$N_{\text{spores,contact,assem}}$	Number of spores on contact material	spores	$=\text{Round}\left(\frac{10^{ContactAssem} \cdot S_{Contact,assem}}{25}, 0\right)$	
$C_{\text{Direct,assem}}$	Direct contamination during assembly (1 →3)	$\frac{\text{spores}}{\text{batch}}$	$=\text{RiskBinomial}\left(N_{spores,contact,assem} \cdot Nr_{Contacts,assem}, Tr\right)$	
Part 2: Contamination due to **indirect contact** from the environment (e.g walls, machines,etc.)				
$NonContactAssem$	Contamination on the environment (non-contact) materials during assemblyof the products	$\frac{\log \text{spores}}{25cm^2}$	RiskNormal(-0.67, 1.34)	Based on data in Chapter 2

Continued on next page

Table 7.8 – continued from previous page

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
$S_{environ,assem}$	Total surface of walls, floors, etc in assembly and packaging environment. Room of 3x3x3m	m^2	54	Expert opinion
$V_{air,assem}$	Volume of the room where assembly and packaging takes place. Room of 3x3x3m	m^3	27	
S_{batch}	Surface of batch of IP j susceptible to recontamination	m^2	1	Company info
t_{assem}	Time exposed to the environment in assembly and packaging zone of IP j	hr	RiskPert(0.25, 0,5, 3)	
k_i	Transferrate coefficient from source (=surface) to air	1/h	RiskUniform (0.01, 10)	den Aantrekker *et al.* (2003)
k_p	Transferrate coefficient from air to batch	m/h	RiskUniform (0.01, 200)	
M_{batch}	Weight of a batch op IP j (same as in module 1)	kg	300	Company info
$C_{source,assem}$	Contamination of source (=surfaces)	Spores/m^2	$=10^{NonContactassem} \cdot \frac{1000}{25}$	

Continued on next page

Table 7.8 – continued from previous page

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
$K_{\text{i,assem}}$		1/h	$= \frac{S_{environ,assem} \cdot k_i}{V_{air,assem}}$	den Aantrekker *et al.* (2003)
$K_{\text{p,assem}}$		1/h	$= \frac{S_{batch} \cdot k_p}{V_{air,assem}}$	
$C_{\text{p,assem}}$	Contamination of batch of IP j per square meter	$\frac{\text{Spores}}{m^2}$	$= K_{i,assem} \cdot C_{source,assem} \cdot [t_{assem} + \frac{1}{K_{p,assem}} \cdot \left(exp\left(-K_{p,assem} \cdot t_{assem}\right) - 1\right)]$	den Aantrekker *et al.* (2003)[b]
$C_{\text{Indirect,assem}}$	Indirect contamination during assembly and packaging of IP j	$\frac{\text{spores}}{\text{batch}}$	$=\text{Round}(C_{p,assem} \cdot S_{batch}, 0)$	
Part 3: Adding both types of recontamination to calculate total recontamination				
N_{assem}	Total recontamination due to assembly and packaging of a batch op IP j	$\frac{\text{spores}}{\text{batch}}$	$= C_{Indirect,assem} + C_{Direct,assem}$	

[a] If applicable

Table 7.9: Parameters, distributions and models used in module 8: Inactivation during pasteurisation of packaged products IP= j:1→3

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
HTT_{pasteur}	Temperature of the pasteurisation treatment	°C	90	Company info
HT, t_{pasteur}	Time of the pasteurisation treatment	min	20	
T_{ref}	Reference temperature	°C	120	van Asselt & Zwietering (2006)
z	Thermal death constant	°C	12.8	
$\log(D_{\text{ref}})$	D-value	min	Same value as module 3 sampled from: RiskNormal(-1.38, 0.56)	
D_{j}	D-value at HTT_{pasteur}	min	$= 10^{D_{ref}} \cdot 10^{\left(\frac{-\left(HTT_{pasteur} - T_{ref}\right)}{z}\right)}$	Holdsworth (2004)
p_{j}	Probability that a spore survives the pasteurisation treatment	-	$= 10^{\left(\frac{-HT, t_{pasteur}}{D_j}\right)}$	
N_0	Spores present in the product before pasteurisation	$\frac{\text{spores}}{\text{batch}}$	$= N_{init} + N_{hand}$	(output module 6 and 7)
$N_{\text{s,pasteur}}$	Spores surviving the pasteurisation	$\frac{\text{spores}}{\text{batch}}$	=RiskPoisson$(N_0 \cdot p_j)$	Nauta (2001) [b]

[a] If applicable

[b] Number of spores surviving the pasteurisation described by a Binomial distribution (N_0, p_j) (Nauta, 2001) and simplified by a Poisson distribution as N_0 is large and p_{j} small.

Table 7.10: Time and temperature distribution during the seven stages of the shelf life

k	Description	$time_k$ (days)	Reference	T_k (°C)	Reference
1	Internal storage at producer	RiskPert(1, 3, 6)	Company info	RiskPert(0, 2.5, 5)	Company info
2	Transport from producer to retail depot	RiskPert(2/24, 0.5, 2)	Company info	RiskPert(1, 3, 7)	Company info
3	Storage at retail depot	RiskPert(2/24, 0.5, 2)	Company info	RiskPert(1, 2.5, 7)	Company info
4	Transport from retail depot to retail store	RiskPert(2/24, 0.5, 1)	Company info	RiskPert(1, 3, 7)	Company info
5	Storage at retail store 80% is consumed in the first 2/3 of the possible selling period, 20% in the last third.	$\begin{cases} X_1 = \text{Riskuniform}\left(0, \frac{2}{3} \cdot \left(SBD - \sum_1^4 time_k\right)\right) \\ X_2 = \text{Riskuniform}\left(\frac{2}{3} \cdot \left(SBD - \sum_1^4 time_k\right), \left(SBD - \sum_1^4 time_k\right)\right) \\ time_5 = \text{RiskDiscrete}\left(\{X_1, X_2\}, \{80\%, 20\%\}\right) \end{cases}$	Nauta (2001)	RiskPert(0, 3, 10.4)	FASFC[a]
6	Transport from retail store to consumer	RiskPert(10, 35, 90) [minutes]	expert opinion	RiskPert(2, 9, 17)	The Weather Channel (2012)
7	Storage in consumer fridge	TTC-distribution[b]	Chapter 6	RiskNormal(6.68, 2.8,Risktruncate(-1,17))	De Vriese *et al.* (2005)

[a] Federal Agency for Safety of the Food Chain (2004),
data gathered as part of an on-going Belgian surveillance plan to assess conformity with respect to retail fridge temperatures

[b] Time to consumption (see 6)

Table 7.11: Parameters, distributions and models used in module 9: growth during the shelf life of the product

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
Part 1: declaration of **batch properties** / General calculations				
$N_{\text{s,pasteur}}$	Spores surviving the pasteurisation	$\frac{\text{spores}}{\text{package}}$	Output of module 8	
M_{package}	Weight of a package	g	450 (= module 7)	Company info
HTT_{pasteur}	Temperature of the pasteurisation treatment	°C	90 (= module 8)	
HT, t_{pasteur}	Time of the pasteurisation treatment	min	10 (= module 8)	
a_w	a_w of the product	-	0.990	Chapter 2
pH	pH of the product	-	5.95	
ε	Error on the lag model	-	Same as module 4, Sampled from: RiskNormal(0, 1.36)	
SE	Error on the growth model	-	Same as module 4, Sampled from: 0.05	
Calculate: $f_2(pH)$; $f_3(a_w)$; $f_4(HT_{\text{pasteur}})$			Formulas: same as table 7.5	
$\prod_i^k \gamma_{i,partial}$			$= \begin{cases} \prod_i^k \gamma_{i,partial} = f_2(pH) \cdot f_3(a_w) \cdot f_4(HT_{pasteur}) & \text{if } pH \leq pH_{opt} \\ \prod_i^k \gamma_{i,partial} = f_2(pH) \cdot f_3(a_w) & \text{if } pH > pH_{opt} \end{cases}$	
Calculate: $\gamma(a_w); \gamma(pH); \varphi(a_w); \varphi(pH)$			Formulas: same as table 7.5	
T_{k}	Temperature during storage step k (k: 1→7)	°C	Table 7.10	
$time_{\text{k}}$	Time of storage step k (k: 1→7)	h	Table 7.10	
SL	Shelf life	days	28	Company info
SBD	Sell By date	days	=SL-Riskuniform(1,3)	expert opinion

Continued on next page

Table 7.11 – continued from previous page

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
$N_{\text{max,package}}$	Maximum number of *B. cereus* in a package	$\frac{\text{CFU}}{\text{package}}$	$N_{max,package} = M_{package} \cdot 10^{N_{max}}$	
Part 2: Division of *B. cereus* based on **psychrotrophic nature**, according to data by Samapundo *et al.* (2011b) $= N_{s,\geq X°\text{C}}$ with X°C the minimal growth temperature				
$N_{(\text{s},\geq 11°\text{C})}$	Number of *B. cereus* only able to grow at temperature of 11°C and up	CFU	=Riskbinomial($N_{s,pasteur}$, 0.121)	
$N_{(\text{s},\geq 10°\text{C})}$	Number of *B. cereus* only able to grow at temperature of 10°C and up	CFU	=Riskbinomial($N_{s,pasteur}$, 0.503) $- N_{s,\geq 11°\text{C}}$	
$N_{(\text{s},\geq 9°\text{C})}$	Number of *B. cereus* only able to grow at temperature of 9°C and up	CFU	=Riskbinomial($N_{s,pasteur}$, 0.938) $- N_{s,\geq 11°\text{C}} -$ $Ns, \geq 10°\text{C}$	
$N_{(\text{s},\geq 8°\text{C})}$	Number of *B. cereus* only able to grow at temperature of 8°C and up	CFU	=Riskbinomial($N_{s,pasteur}$, 0.974) $- N_{s,\geq 11°\text{C}} -$ $N_{s,\geq 10°\text{C}} - N_{s,\geq 9°\text{C}}$	
$N_{(\text{s},\geq 0°\text{C})}$	Number of *B. cereus* able to grow below 8°C	CFU	$=N_{s,pasteur} -$ $(N_{s,\geq 11°\text{C}} + N_{s,\geq 10°\text{C}} + N_{s,\geq 9°\text{C}} + N_{s,\geq 8°\text{C}})$	
Part 3: calculations for stage k: $1 \rightarrow 7$				
Calculate $\gamma(T_{\text{k}})$;$\varphi(T_{\text{k}})$ and $\xi(T_1)$			Formulas: same as module 4	
$f_1(T_{\text{k}})$	Effect of storage temperature on the lag time		Formulas: same as module 4	
$Lag_{\text{calc,k}}$	Calculated lag time during k^{th} stage of storage	days	$lag_{calc,k} =$ $exp\left(a_1 \cdot \prod_i^3 \gamma_{i,partial} \cdot f_4(T_k) - 1 + \varepsilon\right) - 1$	Chapter 5

Continued on next page

Table 7.11 – continued from previous page

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
lag_{k}	Lag time during during k$^{\mathrm{th}}$ stage of storage corrected for model accuracy	hr	$\begin{cases} lag_k = lag_{calc,k} \cdot 24 & \text{if } lag_{calc,k} \geq 1 \\ lag_k = 0 & \text{if } lag_{calc,k} < 1 \end{cases}$	expert opinion
W_{k}	Work to be done' given product properties and storage conditions in k$^{\mathrm{th}}$ stage before growth is possible	-	$W_k = lag_k \cdot \mu_k$	
D_{k}	Work done' during k$^{\mathrm{th}}$ stage of storage	-	$D_k = \mathrm{time}_k \cdot \mu_k$	
W_{k}'	Work to be done' corrected for the work that has been done during the previous stage(s) For stage 1: $W'_k = W_k$		$W'_k = W_k - \sum_0^{k-1} D_k$	
Lag_{k}'	Lag time during k$^{\mathrm{th}}$ stage of storage corrected for the work that has been done during the previous stage(s). For stage 1: $Lag'_k = Lag_k$	hr	$=$ If $\left(W'_k < 0; 0; \frac{W'_k}{\mu_k}\right)$	
TFG_{k}	Time for growth during k$^{\mathrm{th}}$ stage of storage For stage 1: TFG_1 = if $((time_1 - lag_1) > 0; time_1 - lag_1; 0)$	hr	$=$ If $\left[TFG_{k-1} > 0; time_k;\ \text{if } \left(\left(time_k - Lag'_k\right) > 0; time_k - Lag'_k; 0\right)\right]$	
μ_{opt}	Growth rate under optimal conditions	ln/h	see Table 7.5	www.symprevius.net

Continued on next page

Table 7.11 – continued from previous page

Parameter	Description	Unit	Value / Distribution/ Model	Reference [a]
μ_k*	Growth rate during the kth stage of storage	ln/hr	$= \mu_{opt} \cdot \gamma(a_w) \cdot \gamma(pH) \cdot \gamma(T_k) \cdot \xi_{T_k}$	
μ_k	Growth rate during the kth stage of storage with error	ln/hr	$= \text{Risknormal}\left(\sqrt{\mu_k*}, SE\right)^2$	Membré *et al.* (2005)
$N_{(k,\geq X°C)}$	*B. cereus* count after the kth stage of storage and able to grow at a certain temperature For $N_{(1,\geq x°C)}$: $N_{(k-1,\geq x°C)}$= $N_{(s,\geq x°C)}$	CFU	$\begin{cases} N_{(k,\geq x°C} = 0, \text{ if } k-1, \geq x°\text{C} = 0 \\ N_{(k,\geq x°C} = N_{k-1,\geq x°C}, \text{ if } T_k < x°\text{C} \\ N_{(k,\geq x°C} = Round\left(\left(\frac{N_{max,package}}{1+\left[\left(\frac{N_{max,package}}{N_{k-1,\geq x°C}}-1\right)\cdot exp(-\mu_k \cdot TGK_k)\right]}\right); 0\right) \\ \text{if } N_{k-1,\geq x°C} > 0 \text{ and } T_k \geq x°\text{C} \end{cases}$	Rosso *et al.* (1996)
N_k	*B. cereus* count in the package after the kth stage of storage	CFU	$= \sum_{(x\geq 0°C)}^{(x\geq 11°C)} N_{(k,\geq x°C)}$	

[a] If applicable

7.4 Results and discussion

7.4.1 General model output

An example of the model output at the moment of consumption is given in figure 7.3. The red bars represent all the packages that contain at least 1 *B. cereus* spore or cell. The green bar represents packages that contain no *B. cereus* and the orange bar represents packages that are not consumed, but discarded because they have exceeded their 'use by' date (including the margin taken by the consumer). The 'x' to the right of the red distribution is the location of the packages that contain the emetic toxin. The bar itself is to small to be visible.

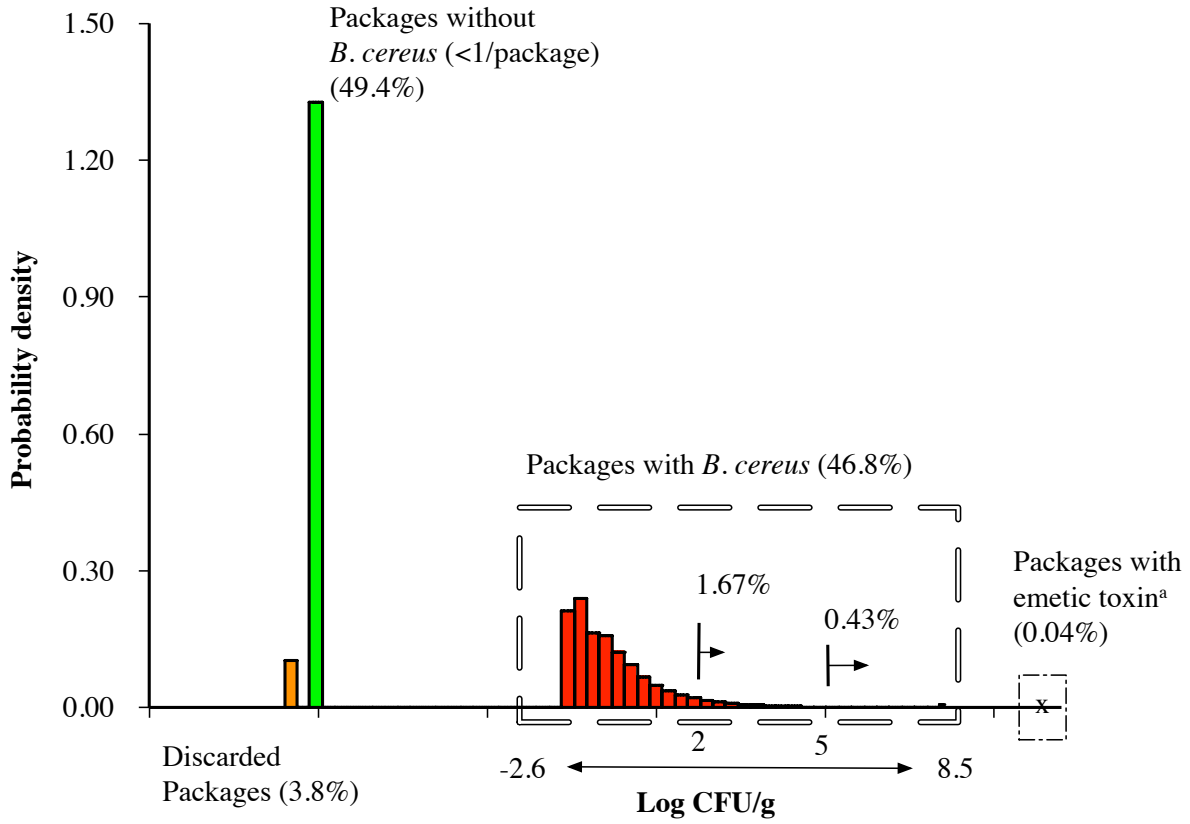


Figure 7.3: Distributions of the *B. cereus* concentrations and prevalence in REPFEDs at the moment of consumption

As indicated on the figure, the *B. cereus* concentration varies between -2.6 log CFU/g (1 *B. cereus* spores per 450g) and 8.5 log CFU/G (N_{max}). The distribution is skewed to the left and

has a long tail towards the higher concentrations. The percentage of packages that contains a *B. cereus* concentration that is detectable by classical plate counting (i.e. $\geq 10^2$ CFU/g) is 1.67% or 16,700 per 10^6 packages. The percentage of packages with a too high *B. cereus* concentration (i.e. $\geq 10^5$CFU/g) is 0.43% or 4313 packages per 10^6 packages. An additional 0.04% of packages (437 packages per 10^6 packages) can contain the emetic toxin. This brings the total number of 'risky' packages at 0.475%, i.e. 4750 per 10^6 packages or 1 package per 210 packages.

7.4.1.1 Prevalence

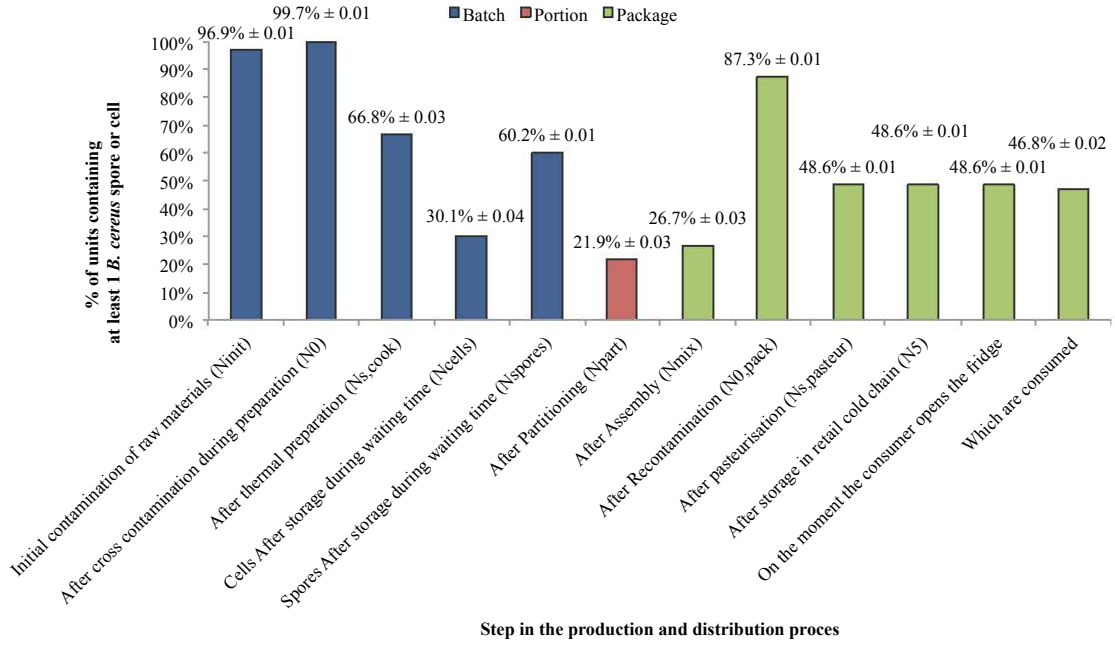
Figure 7.4a shows the evolution of prevalence throughout the production process and shelf life. During the first two production steps (raw materials and cross contamination during handling), the prevalence is high (96.9 - 99.7%). Although this may seem very high, prevalence at this stage of production corresponds to a very low concentration (1 spore/batch = 1/400kg = -5.6 log spores/g). There is a first reduction (±33% 99.7%→66.8%) in *B. cereus* spore prevalence during the thermal preparation process. A second reduction in spore prevalence takes place during intermediate storage after cooking. Although there is no inactivation, so no actual decrease of prevalence during storage, spores may germinate and this reduces prevalence of spores. During the subsequent partitioning there is another decrease in prevalence. In this process, the large batches (400kg) are divided in small portions (150g). Since a large percentage of batches contained only a low level of *B. cereus* spores (50% of the contaminated batches contained less than 250 spores (per 400kg)), some portions are not contaminated after partitioning. When the three portions are put in the tray there is a slight increase in prevalence, since each assembled product has three chances of being contaminated (i.e. one per intermediate product). The fact that there is only a slight increase in prevalence may appear counterintuitive. If the prevalence after partitioning (p) is 21.9% than it would be expected (for three independent portions) that the prevalence after mixing is 53.4% ($= 1 - (1 - p)^3$). However, the three portions are not independent since they are produced in the same environment. Therefore, the probability of recontamination during handling of raw materials is similar for each of the three product components.

The first major increase in prevalence occurs during the packaging. In this stage the prevalence increases more than threefold from 26 to 87%. Although, this increase seems very high, based on the size of the change in median spore concentration (-1.5→-1.4 log CFU/g) or even in the 99th percentile of the spore concentration (3.2→2.2 log CFU/g) (Table 7.12), it is clear that the recontamination only adds a small number of spores per package. The decrease in median spore concentration due to recontamination seems counter intuitive but is due to the fact that most product that are recontaminated will only contain a low concentration of *B. cereus*, shifting the

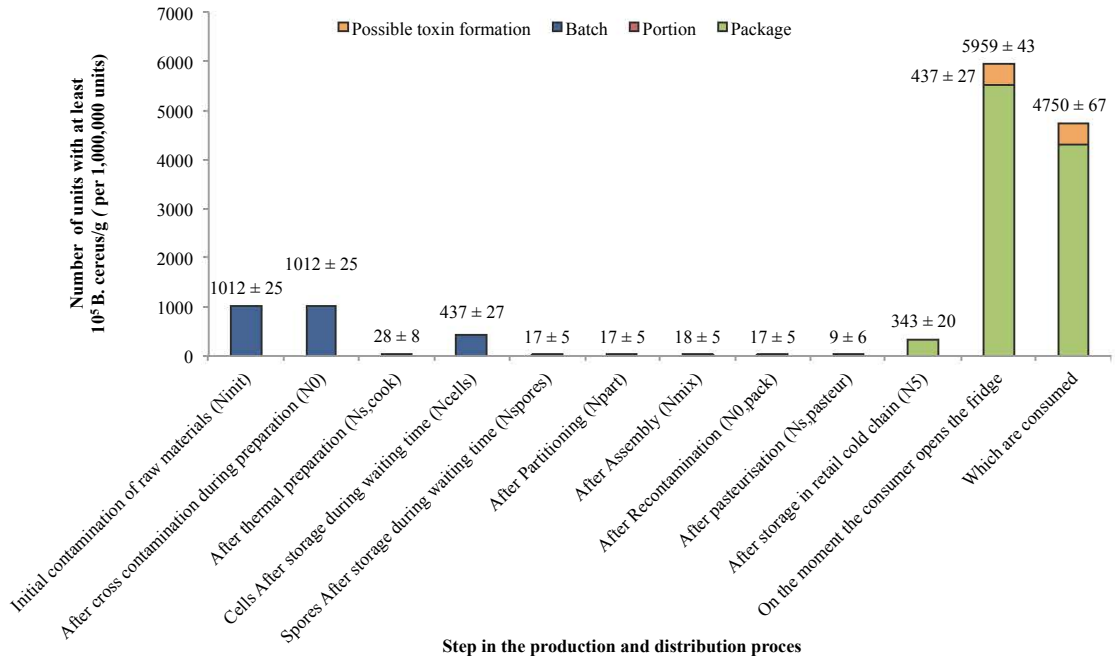
concentration distribution to the left. The last reduction in prevalence occurs during pasteurisation. This thermal step does not only decrease the prevalence, but also has a significant effect on the concentration. Since the packages are sealed, there is no further change in *B. cereus* prevalence.

Theoretically, the prevalence can still change during shelf life, if (i) a package is leaking, or if (ii) *B. cereus* dies off. If a package leaks, recontamination is possible and the prevalence could increase. A decrease of prevalence is also theoretically possible if the *B. cereus* spores/cells die during storage. Because there was insufficient data about these processes, they were not included in the exposure assessment model.

Three processes seem to be crucial to the change in prevalence during processing: (i) Raw material contamination, (ii) recontamination during packaging (iii) thermal preparation and pasteurisation. The first two cause high prevalence, the last is needed to reduce prevalence in the final product.



(a) Prevalence



(b) Units with more than 10^5 *B. cereus*/g

Figure 7.4: (a) Prevalence (≥1 *B. cereus* spores/unit) and (b) number of units (batches, portions or packages) with a *B. cereus* concentration ≥ 10^5 CFU/g (for 10^6 iterations) throughout the REPFED production and shelf life. (± Standard deviation based on 3 simulations). Different colours represent different unit sizes.

Table 7.12: Overview of the model output per module: prevalence (≥1 *B. cereus* per unit), median and 99th percentile of *B. cereus* counts, percentage of units exceeding 10^5 CFU/g. Data are the average values of 3 simulations (each 10^6 iterations) with their standard deviation (n=3).

Step in the production process	Prevalence (%±sd.[a])	*B. cereus* count (log CFU/g ±sd.).		% of units with more than 10^5 *B. cereus*/g
		Median	99th perc.	
Initial contamination of mix	96.9% ± 0.01	-1.5 ± 0.002	4.0 ± 0.007	0.1% ±0.002
After recontamination during preparation	99.7% ± 0.01	-1.6 ± 0.001	3.9 ± 0.006	0.1% ±0.002
After cooking	66.8% ± 0.03	-2.6 ± 0.001	3.0 ± 0.002	0.003% ±0.001
Cells after storage during waiting time	30.1% ± 0.04	-2.1 ± 0.002	3.7 ± 0.012	0.04% ±0.003
Spores after storage during waiting time	60.2% ± 0.01	-2.9 ± 0.004	2.7 ± 0.007	0.002% ±0.001
After partitioning during packaging	21.9% ± 0.03	-1.1 ± 0.02	3.3 ± 0.005	0.002% ±0.001
After assembly during packaging	26.7% ± 0.03	-1.5 ± 0	3.2 ± 0.003	0.002% ±0.001
After recontamination during packaging	87.3% ± 0.01	-1.4 ± 0	2.2 ± 0.008	0.002% ±0.001
After pasteurisation (leaving factory)	48.6% ± 0.01	-1.7 ± 0	2.4 ± 0.006	0.001% ±0.001
After storage in retail cold chain (moment of purchase)	48.6% ± 0.01	-1.7 ± 0	2.5 ± 0.009	0.03% ±0.002
Which are eaten	46.8% ± 0.02	-1.7 ± 0	4.7 ± 0.049	0.43% ±0.005

[a] Standard deviation (n=3)

7.4.1.2 Number of units with ≥ 10^5 spores or cells /g

Figure 7.4b shows the number of units containing at least 10^5 spores (or cells) per g during production and shelf life. A significant percentage of initial raw material mix (H_0) contained too high counts of *B. cereus* (0.1% or ca. 1000 in 10^6 batches). This number is likely to be an overestimation caused by the uncertainty in raw material contamination and the assumption that all raw materials are homogeneously contaminated. Because *B. cereus* is likely present as spores this is not necessarily a problem at this stage. However, the high counts at this stage increase the burden on the downstream processing to reduce the *B. cereus* counts. The high contamination of raw materials also means a huge potential of introduction *B. cereus* on equipment. This can enable *B. cereus* to become 'resident flora' with the ability to form persistent biofilms. This stresses the importance of supplier selection for raw materials.

Although the primary objective of the first thermal preparation (cooking or baking) is not to inactivate microorganisms, it has a considerable effect on the number of batches containing high counts. The number of batches with more than 10^5 CFU/g decreases by 98% to 0.003% (28 in 10^6 batches). During intermediate storage after pasteurisation, spores can germinate to cells and start growing. This process has a dual effect. Spores that germinate are eventually inactivated during pasteurisation and are no longer a risk. However, the cells that originate from these germinated spores can start growing and if their concentration exceeds 10^5 CFU/g there is a risk of toxin formation. The results show that in 17 batches there was little or no germination and that the high concentration of spores remained present. In 437 batches the cell concentration exceeded 10^5 CFU/g. In most (±75%) of the cases this was due to a high raw material contamination (min. 10^4 CFU/g) combined with a limited inactivation during cooking. In the other cases, the product was already moderately contaminated (10^3 CFU/g) and was stored for 3-4 hours at temperatures between 20 and 40°C. During the rest of the production process, the *B. cereus* concentration in these batches (with high cell count) may decrease considerably, but because the emetic toxin is heat stable (Rajkovic *et al.*, 2008) these batches remain a risk and hence all the products that are made from these batches, are also a risk. This is indicated on figure 7.4b as the orange coloured bars in the last two columns.

Until pasteurisation, the number of products with high spore counts or high cell counts remain constant. There is slight reduction during pasteurisation (-8 packs per 10^6), and a significant increase (9→ 334 packages per 10^6) during storage in the retail, but the most significant increase occurs during consumer storage (343→ 5522 packages per 10^6). Analysis of the individual iterations showed that this was due to three causes: (i) high concentration present in the finished product (ii) excessively long storage times and (iii) temperature abuse during home storage.

However, luckily not all these packs are consumed. In the 'Time to Consumption' approach used for consumer storage (See chapter 6), there is an additional check by the consumer, to see if the product has exceeded its 'use by' date. Depending on the type of consumer, these products can be eaten or discarded. In the latter case, these products are not considered to be a risk. When the results of the final products were analysed more in depth, an interesting link was found between a consumers respect for the 'use by' date and the exposure to high *B. cereus* counts (Table 7.12). Consumers that strictly respected the 'use by' date purchased 52.5% of the (simulated) REPFEDs but only consumed 41.5% of the REPFEDs with a concentration or 10^5 CFU/g or higher. For consumers with '*no respect*' for the 'use by' date, the ratio is inversely skewed. These consumers accounted for only 4.7% of the REPFED purchases in the model, but they consumed 7.5% of the products with high *B. cereus* concentrations. The effect of respect for the 'use by' date on the exposure was found to be statistically significant (χ^2=279.4, df=3, p<0.01).

Temperature in consumer fridge was also found to be a crucial factor. Of the packages exceeding 10^5 CFU/g at the moment of consumption, 86.4% (±0.77%) was found in iterations with a refrigerator temperature higher than 8°C. Consumers with a fridge temperature higher than 8°C have between 12.8 and 14.6 times the odds of consuming a product with more than 10^5 *B. cereus* per g. This means that temperature abuse is a critical factor in the exposure to *B. cereus* from REPFEDs. Rajkovic *et al.* (2005) also reported a large effect on growth over a small range of temperature. They reported that at 7°C, growth of one *B. cereus* was at least partially inhibited by the rapid growth of *Bacillus circulans*, while at 10°C the opposite was true. It has previously been reported that outbreaks of emetic *B. cereus* intoxication are usually (though not exclusively) linked to heat treated foods that were not stored at refrigeration temperature (EFSA, 2005a).

Nauta (2001) reported that the *B. cereus* concentration at the end of the production process, was a bad predictor for the final *B. cereus* concentration. To test whether this was the case in the current QMEA, the *B. cereus* concentration in the final product ($N_{s,pasteur}$), was plotted against the *B. cereus* concentration after consumer storage (N_7). Figure 7.5 demonstrates that the *B. cereus* concentration of the final product is not a good predictor for the concentration after storage in the cold chain.

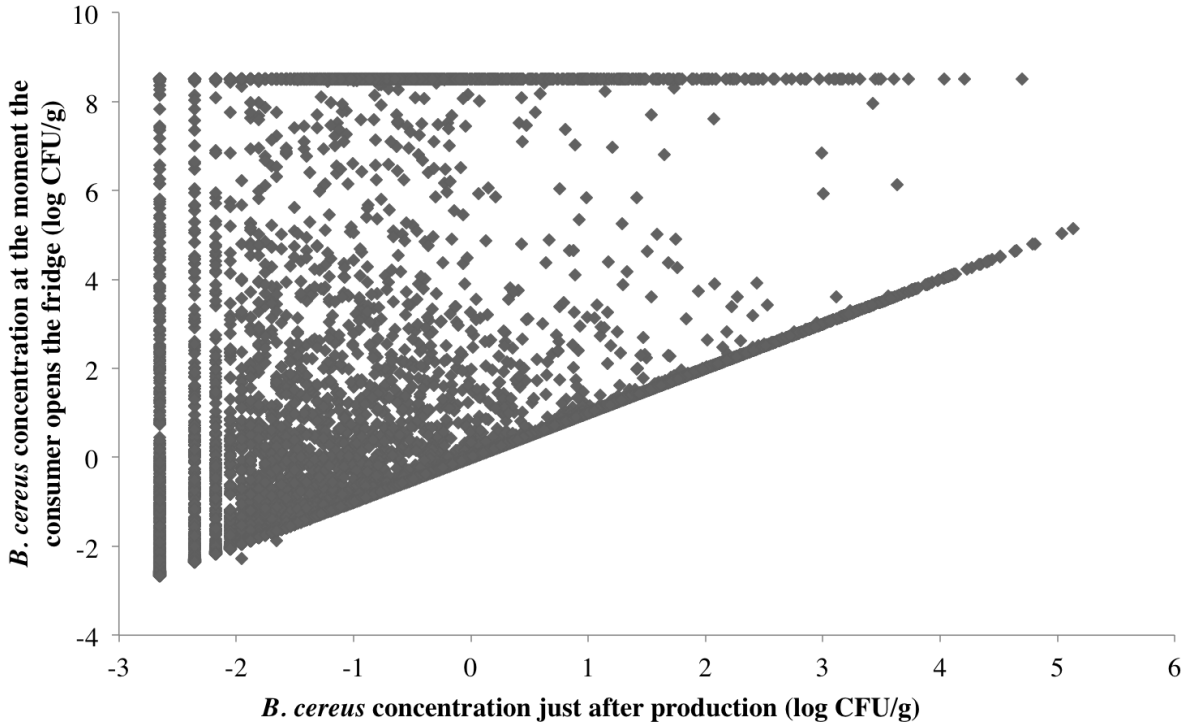


Figure 7.5: Plot of the *B. cereus* concentration at the moment the consumer opens the fridge, as a function of the *B. cereus* concentration immediately after the production process.

7.4.2 Sensitivity analysis and convergence of model predictions

To determine which parameters had the most effect on the output a tornado plot was created. This plot shows the difference between model output when a distribution is fixed at its lowest (1st percentile) and highest value (99th percentile). All stochastic parameters in the model were tested, but only the ten parameters with the largest impact on the model outcome are given in Figure 7.6.

The tornado plot shows that consumer behaviour (storage time and temperature); strain variability and modelling error (error on growth/lag model and variability in D-value) are the most significant drivers of changes in model output. The variability caused by consumer behaviour is a mix of variability and uncertainty, meaning that more research or more measurements will still reduce the variability of the output, but that some variability is inherently present and will remain. The variability in model output caused by strain variability is also a mix of both. Using a more accurate model (e.g. built with more relevant data) will reduce the modelling uncertainty, but the strain variability in *B. cereus* vegetative cell growth and spore lag time will remain important. Besides consumer behaviour and strain variability, recontamination during packaging,

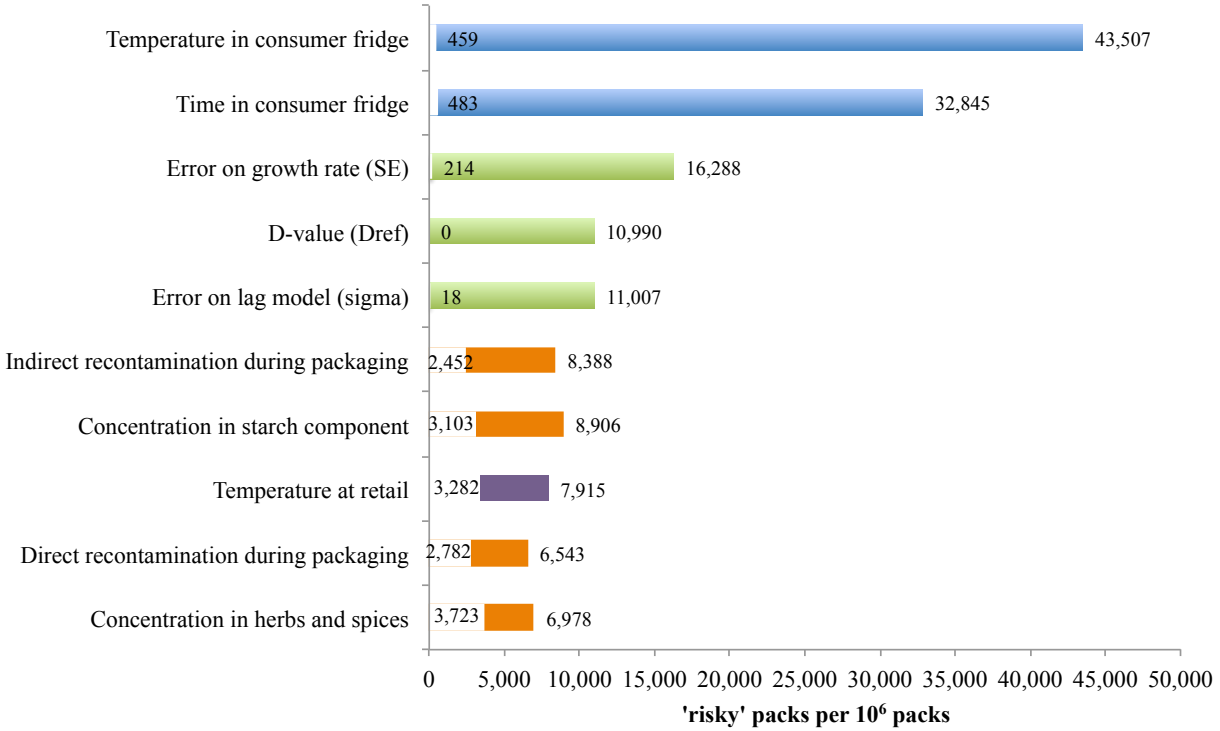


Figure 7.6: Tornado plot of the ten parameters with the largest effect on the final model output (in descending order of effect). Bars show the difference in model output ('risky' packs per 10^6 packs) when the parameters in question is fixed (e.g. temperature in consumer fridge), first at its 1st percentile value (e.g. 0°C), and second at its 99th percentile value (13°C), while all other parameters remain variable. The different colours point to the different responsibilities or sources: blue = consumer behaviour, green = strain variability and modelling error, orange = production process and purple = retail

raw material contamination and retail temperature were important factors.

To test the convergence of the model predictions, the model was run three times and the average and standard deviation of all outputs was determined (Figure 7.4). The standard deviation of the number of packs exceeding 10^5 CFU/g is small: 67 on 4750 (or ± 1%). For the prevalence the convergence was even better: 0.02% on 45.0%.

7.4.3 Validity of model estimates

To evaluate the model estimates, a comparison with values found in literature was performed. For prevalence, the result (48.6%) is similar to that reported by Samapundo *et al.* (2011b), who found that 56.3% of the tested food products contained *B. cereus* (both mesophilic and psychrotrophic).

Similarly, Choma *et al.* (2000b) reported that after 4-12 days at room temperature 70% of the tested cooked chilled foods contained *B. cereus* (i.e. both mesophilic and psychrotrophic). After 20 days at 10°C, this was still the case for 50% of the tested products (only psychrotrophic strains). Del Torre *et al.* (2001) reported a prevalence of 33% (all lower than 10^2 CFU/g) at the time of production. Finally, the model by Nauta (2001) predicted a prevalence of 62-100% depending on the strain, with psychrotrophic strains having a lower prevalence. Guinebretière *et al.* (2003) reported a *B. cereus* contamination on pasteurised zucchinis (before storage) of less than 0.2 log CFU/g. In spite of this very low contamination; *B. cereus* was still able to grow to high concentrations (>10^4 CFU/g) at 10°C. However, at the advised storage temperature of 4°C no change in *B. cereus* concentration was noted.

For the number of packs exceeding 10^5 CFU/g at the moment of consumption (0.48%), there is no measured data available, which is probably due do the low probability of finding such samples. In his MPRM-report, Nauta (2001) reported that between 0 and 6.4% of the packages was predicted to exceed 10^5 CFU/g, depending on strain and temperature profile (north vs. south of Europe). In the same report, the percentage of products showing growth during storage at the consumer stage was estimated between 2 and 38%, in the current model this percentage is 7.8% (±0.2).

However, it remains difficult (if not impossible) to validate this type of models using actual data. The frequency of highly contaminated packs is very low and highly dependent on the consumer behaviour, as well as on the *B. cereus* characteristics. For example, in theory it would have been possible to compare the model predictions with the results reported in chapter 2 (Table 2.3, p. 54 and Tables 2.7-2.10, p. 65-68). However, to using these data would require information about the production process (times/temperatures) and the product formulation. In addition virtually all the microbiological data in these tables concerns different products, which means that the model would have to be adapted for each individual product. Below some of these factors of uncertainty and variability are discussed in detail.

7.4.3.1 *B. cereus* characteristics

Despite our best efforts, a model is per definition an approximation of a more complex reality. While a significant amount of variability/uncertainty was included in the model (e.g. D-values, error on the lag model, consumer behaviour, etc.), there was not always sufficient data/knowledge available. More specifically this remains an issue for *B. cereus*, due to the large inter-strain variability (see section 7.2.2.1). In addition no information is available about the spores:cells ratio present in the different sources of contamination (raw materials, environment). Therefore,

a worst-case approach was used by assuming that only spores were present (see section 7.2.2.1). Finally, it remains unclear which *B. cereus* strains can produce toxins (emetic, diarrhoeal or both). Not all strains have this capacity (Ceuppens *et al.*, 2011). Afchain *et al.* (2008) performed an exposure assessment of *B. cereus* in cooked chilled foods, taking genetic diversity into account. Their conclusion was that group II (psychrotrophic and cause of foodborne illnesses) and group IV (psychrotrophic but not the cause of food borne illnesses) were the most important groups after home storage. However, *B. cereus* from group IV produces very low amounts of enterotoxins. The exposure assessment by Afchain *et al.* (2008) was done for a specific product (courgette purees) and from previous research the distribution of different *B. cereus* groups in these products was known (Guinebretière & Nguyen-The, 2003). The exposure assessment presented in this study is not designed for one single product and no information was available about the distribution of the different *B. cereus* groups in REPFEDs. A worst-case assumption was therefore used: all *B. cereus* strains have the capability to produce both the emetic toxin and the enterotoxins (diarrhoeal).

7.4.3.2 Predictive models

The vegetative cell growth and heat-treated spore lag model used were based on data gathered in laboratory medium and were not validated on cooked chilled products. Since bacteria usually exhibit shorter lag times and higher growth rates in lab media than in food products, the model predictions are presumably worst-case predictions (Faille *et al.*, 1997). Furthermore, spores generated at 10°C and 30°C also have different growth characteristics (Gonzalez *et al.*, 1999; Baril *et al.*, 2012)

7.4.3.3 Packaging and other factors

A considerable amount REPFED products are packed under modified atmosphere (MAP). Our own research suggests that under high CO_2 concentrations (±30%) and low O_2 concentrations (<1.5%) the minimum growth temperature of psychrotrophic *B. cereus* strains increased from 7 to 9°C (Unpublished). Samapundo *et al.* (2011a) noted similar observations for *Bacillus weihenstephanensis*, with decreased growth rates and lower maximum population density obtained for storage at low temperatures under MAP. In contrast, several studies have shown that a reduced oxygen concentration can stimulate enterotoxin production, and that the bacteria that survive MAP might be more virulent (Duport *et al.*, 2004; Van Der Voort & Abee, 2009). However, this information is still insufficiently quantifiable to use in the exposure assessment.

The current exposure assessment does not consider the effect of competing microorganisms on

B. cereus. Research has shown that growth of lactic acid bacteria can affect the germination and growth of *B. cereus* (Wong & Chen, 1988). However, there is insufficient information (prevalence, model, etc.) to quantify this effect on *B. cereus*. In addition to affecting *B. cereus* growth, the competing flora can also affect product quality (i.e. spoilage). For similar reasons the exposure assessment also does not include the effect of spoilage. It is likely that long storage times in combination with possible temperature abuse at consumer levels will cause spoilage. In some cases the consumer can detect this spoilage and the product will be discarded. Spoilage was not considered in the model for a number of reasons: (i) REPFEDs are a diverse product group, with equally diverse microbial spoilage; (ii) because the time-temperature combinations that lead to spoilage are not yet adequately quantified or at least no available in the public domain; (iii) because *B. cereus* can grow to dangerous concentrations without causing noticeable spoilage (Beattie & Williams, 2002).

7.5 Conclusions

The quantitative microbiological exposure assessment of *B. cereus* shows four key points in the life cycle of a REPFED: (i) Raw material contamination, (ii) recontamination during packaging, (iii) reduction during pasteurisation and (iv) consumer behaviour. The first key point can be controlled using supplier selection and compliance testing. Additional cleaning and disinfection and/or reducing exposure time can improve the second key point. The third point, pasteurisation is already a critical control point in the REPFED production process. The fourth and final key point is difficult to control, and this shows the importance of general consumer education about food storage and the importance of respecting the 'use by' date. This consumer behaviour is the primary reason that end-product concentration is not a good predictor for the concentration after storage in the cold chain. The presented model will be further explored via scenario analysis (Chapter 8) and iso-risk curves (Chapter 9), to evaluate potential strategies for REPFED producers to reduce the heat treatment during pasteurisation and improve the sensorial quality of REPFEDs.

Chapter 8

A Quantitative Microbiological Exposure Assessment of *B. cereus* in REPFEDs: Part 2 - Scenario analysis

Summary

In this chapter, sixteen scenarios are evaluated, which are based on the QMEA model presented in chapter 7. The scenarios are designed to determine the impact of a specific processing step (e.g. pasteurisation), a specific type of consumer behaviour (e.g. consuming after 'use by' date), or model assumptions (all *B. cereus* produce toxins) on the number of 'risky' packages ($\geq 10^5$ CFU/g or presence of cereulide). The scenarios demonstrate that the exposure to *B. cereus* from REPFEDs is the responsibility of both producers and consumers. On a producer level it is important to maintain good hygiene, especially during packaging and assembly. Loss of control at this stage of processing is detrimental to product safety (exposure $\times 5$ to 24,291 packages per million). On a consumer level, it is crucial set the refrigerator at the correct temperature (≤ 7 °C). If all consumer refrigerators would be at the correct temperature, this would reduce exposure by approximately 80% (to 929 packages per million).

8.1 Introduction

In the previous chapter, a quantitative microbiological exposure assessment (QMEA) for *B. cereus* in in-pack-pasteurised REPFEDs was presented. The QMEA, further referred to as the 'baseline scenario' offered a first insight in the current prevalence, concentration and toxin formation during the production and shelf life of REPFEDs. The primary output of this QMEA was the number of 'risky' packages per million REPFEDs. A package is considered a risk if the *B. cereus* concentration during shelf life is 10^5 CFU/g (or higher) or if emetic toxin production was possible during the production process. A preliminary **sensitivity analysis** was performed in chapter 7 to determine the impact of **single parameters** (e.g. the distribution of D-values) on the model output. During sensitivity analysis, the model output is compared for the lowest value and the highest value of a parameter (i.e. how sensitive is the model to this parameter). Consumer behaviour (e.g. refrigerator temperature) and strain variability (e.g. D-value) were the most important causes of variability. In the current chapter a **scenario analysis** is performed. During this process, **multiple inputs** (values or distributions) or **assumptions** are changed, and the effect on the output is assessed.

To determine the effect of different processing conditions and possible consumer behaviours, sixteen scenarios were developed, simulated and their output was compared to the baseline scenario of the QMEA presented in chapter 7. The goal was to determine which of the processing steps or types of consumer behaviour had the highest impact on the exposure. This information can be used by risk managers to prioritise different risk mitigation strategies and to determine

where efforts should be made along the production and distribution chain. Fifteen scenarios are straightforward adaptations of the QMEA (e.g. changing the distributions or a parameter). In the sixteenth scenario one of the basic assumptions of the QMEA is changed (section 8.2.1.9).

8.2 Methodology

All the scenarios presented in this chapter use the basic QMEA model presented in Chapter 7 with one or more changed parameter values. The basic model will be referred to as the '**baseline scenario**'. Scenarios were selected by expert discussion based in the results of chapter 7. An overview of the different scenarios and which parameters were changed for each scenario is given in Table 8.1. Each scenario was simulated three times with 1,000,000 iterations and, just as for the baseline scenario, the following five outputs were determined after each module of the QMEA (1→9):(i)% units $\geq 10^5$ *Bacillus cereus*/g; (ii) % units possibly containing cereulide; (iii) % prevalence (i.e. units with at least 1 *B. cereus* cell or spore); (iv) Median *B. cereus* concentration in the contaminated packs (log CFU/g); (v) 99^{th}-percentile of the *B. cereus* concentration in the contaminated packs (log CFU/g).

8.2.1 Description of the scenarios

A total of 16 scenarios (sc. 1-16) was tested, 15 simulating a change to one or more parameters in a specific module of the QMEA and one simulating a change in a basic assumption of the QMEA. Each scenario is discussed in detail below. An overview of the parameters changed per scenario is given in Table 8.1.

8.2.1.1 Scenario 1 - Raw material contamination

This scenario (sc. 1) simulates the effect of a company setting more stringent specifications for *B. cereus* in raw materials. In the baseline model, this specification was set at 10^4 CFU/g. This corresponds to the tolerance value specified for several raw materials in Uyttendaele *et al.* (2010). Because companies cannot sample all batches of each raw material, for economical and practical reasons, it is unlikely that all samples containing more than 10^4 CFU/g will be detected. To account for this uncertainty, the limit in the baseline scenario was increased with 10^2 CFU/g. This puts the original truncation of the raw material contamination (i.e. the maximum simulated value) at 10^6 CFU/g. In the first scenario the specification is lowered to 10^3 CFU/g (the target value according to Uyttendaele *et al.* (2010)). Taking the same uncertainty into account (10^2 CFU/g), this puts the truncation at 10^5 CFU/g.

8.2.1.2 Scenario 2 and 3 - Hygiene during handling of raw materials

In the baseline scenario, the contamination on contact and non-contact materials during handling (module 2 - C_h) was modelled by a normal distribution based on the samples collected in chapter 2. To determine the effect of improved or decreased hygiene at the handling of raw materials, two scenarios were simulated. The first (sc. 2) simulated a best-case hygiene at this level, by fixing the two types of contamination (contact and non contact materials) at the lowest simulated value of the baseline scenario. The second (sc. 3) scenarios simulated the exact opposite: worst-case hygiene. This was simulated by fixing the two types contamination at their highest simulated values.

8.2.1.3 Scenario 4, 5 and 6 - Intermediate storage

After preparation, the product usually has to wait a certain time before processing continues. During this waiting period (Module 4 -G_i), spores that have survived the first heat treatment (e.g. cooking) can germinate and grow. The vegetative cells, germinated from the spores, are eliminated during the subsequent pasteurisation treatment. However, if their concentration exceeds 10^5 CFU/g, toxin formation is possible (see Figure 7.2, p. 168). To determine which time and temperature combination of storage is the most suitable three combinations were compared: (sc. 4) 15h at 10°C (insufficiently cold and long), (sc. 5) 8h at 22°C (shorter but still warm) and (sc. 6) 15min at 37°C (very short and warm). These time-temperature profiles were selected based on expert discussion.

8.2.1.4 Scenario 7 and 8 - Hygiene during assembly and packaging of final products

These scenarios are similar to scenarios 2 and 3 in set up, but affect the contamination during assembly and packaging (module 7 - C_a). Scenario 7 simulates best-case hygiene (i.e. very low environmental contamination) and scenario 8 simulates worst-case hygiene.

8.2.1.5 Scenario 9 and 10 - Alternative pasteurisation treatments

To determine to what extent a deviation from the standard safe harbour pasteurisation treatment (P_{90} =10 min) will affect the risk, two scenarios were tested. Each simulates a different pasteurisation treatment (module 8 - R_p). Scenario 9 simulates a pasteurisation of 10 minutes at 87°C, and scenario 10 simulates 5 min at 95°C.

8.2.1.6 Scenario 11 - Shorter shelf life

To determine the effect of the shelf life, it was shortened from 28 days to 21 days.

8.2.1.7 Scenario 12, 13 and 14 - Improved respect for the 'use by' date

In chapter 6 it was demonstrated that a longer shelf life not necessarily means that the product is stored for longer times (Table 6.6, p. 151). At least not for the majority of the products, no matter how long the shelf life stated on the label, 93% was consumed within 7 days after purchase. However, if the shelf life is shorter, more products are discarded instead of consumed. The QMEA model uses the 'time to consumption' (TTC) approach (Chapter 6). In this approach, it is first determined how long a product is stored in the fridge (e.g. 15 days). This time is then compared to the shelf life left at the time the consumer buys the product (remaining shelf life = Shelf life - time in retail). If the storage time in the fridge is longer than this remaining shelf life, the product has passed the 'use by' date. However, not all consumers will discard the product. Depending on the consumer's respect for the 'use by' date a certain margin will be taken. For '*strict*' consumers it is assumed that no margin is taken. In other words the product is discarded once it has passed the 'use by' date. Consumers with '*moderate*', '*little*' or '*no*'-respect will respectively take a margin of 3, 7 and '∞' days on the 'use by' date. Because '∞' is impossible to use in simulations, this value was set at 60 days.

Each of the three scenarios excludes one or more type(s) of consumer behaviour. The margin taken by the consumers in the excluded groups is set to the margin taken by the consumers of the group with the worst' behaviour still included in the model. For example: scenario 12, does not include the '*no*'-respect consumers. So all consumers with this behaviour are now transferred to the '*little*'-respect group. Therefore, It is assumed that they now take 7 instead of ∞ days of margin on the 'use by' date. Scenario 13 does not include the '*no*' or the '*little*' group, so these are now assumed to be in the '*moderate*' group. Finally, scenario 14 assumes that all consumers are '*strict*' consumers.

8.2.1.8 Scenario 15 - Only 'reasonable' fridge temperatures

Most REPFEDs are designed to be stored at 4°C and this is usually indicated on the label (e.g. "store at maximum 4°C"). Unfortunately, the temperature in consumer fridge is very variable (Derens *et al.*, 2004; De Vriese *et al.*, 2005). Article 3 of Regulation (EC) No 2073/2005 states: "*Food business operators shall ensure (. . .) that the food safety criteria applicable throughout the shelf life of the products can be met under reasonably foreseeable conditions of distribution, storage and use*". However, the question remains what is 'reasonable foreseeable abuse'. The

technical guidance document on shelf life studies for *L. monocytogenes* in ready-to-eat foods (EU CRL for *Listeria monocytogenes*, 2008) states that for challenge tests, the 75th percentile of the temperature should be used. In Belgium this corresponds to 8°C (De Vriese *et al.*, 2005; Vermeulen *et al.*, 2011). For this scenario it was assumed that temperatures exceeding 8°C were not 'reasonable' and they were therefore excluded in this scenario. This was achieved by lowering the right side truncation (i.e. maximum simulated temperature) of the distribution of consumer fridge temperatures, from 17 to 8°C.

8.2.1.9 Scenario 16 - Not all *B. cereus* strains can produce emetic toxins

This scenario is fundamentally different from the other fifteen, because it does not simply change a parameter in the QMEA. In this scenario, one of the more fundamental assumptions in chapter 7 is changed. It is currently not possible to identify which *B. cereus* strains are likely to be present, nor if multiple strains will be present, nor how many different strains will be present (in the raw materials or in the production environment). Therefore, a simple assumption was used in the baseline model: It was assumed that all the *B. cereus* strains present in the REPFEDs had the capacity to produce both diarrhoeal and emetic toxins. While this may be close to reality for the diarrhoeal enterotoxins, it is most likely not the case for the emetic toxins (Samapundo *et al.*, 2011b; Carlin *et al.*, 2006; Altayar & Sutherland, 2006; Ceuppens *et al.*, 2011).

Hence, in this scenario the assumption set out in section 7.2.2.1 is abandoned and a new assumption is made. In this scenario it is assumed that 3.2% of the *B. cereus* strains have the capacity to produce emetic toxins (Altayar & Sutherland, 2006). However, because the number of different strains that is present is unknown, a new additional assumption is needed: that all *B. cereus* cells or spores can be from a different strain. This is of course, a worst-case assumption. These two new assumptions cause a fundamental change in the logic of two modules: module 4 (G_i - growth during intermediate storage) and module 9 (G_s - growth during shelf life).

For **growth during intermediate storage** the assumptions are applied as follows:

- All spores that turn into cells can be from a different and potentially emetic strain.
- The number of cells able to grow **and** to produce toxins ($N^*_{0,j}$) is determined using a Poisson distribution as approximation for the Binomial distribution, which gives computational problems in the @Risk software (Table 7.5, p.179).

$$N^*_{0,j} = \text{RiskPoisson}\left(\left(N_{s,cook,j} \cdot \%germ\right) \cdot 0.032\right) \tag{8.1}$$

with $N_{\text{s,cook}}$ the number of spores that survived the heat treatment, $\%germ$ the percentage of spores that is able to germinate.

- This value ($N^*_{0,\text{j}}$) is used to calculate $N_{\text{cells,j}}$. If this value exceed 10^5 CFU/g than there is a risk of toxin presence in the batch.

For **growth during shelf life** the assumptions are applied as follows:

- All spores that turn into cells can be from a different and potentially emetic strain.
- If growth occurs during shelf life (i.e. if the lag time is shorter that the storage time), the number of cells able to produce toxins of those surviving pasteurisation ($\text{N}^*_{\text{s,pasteur}}$) is determined using a Poisson distribution (Table 7.11, p 189). This value is then used for the rest of the calculations.

$$N^*_{s,pasteur} = \text{RiskPoisson}\left(N_{s,pasteur} \cdot 0.032\right) \tag{8.2}$$

  with $N_{\text{s,pasteur}}$ the number of spores that survived the pasteurisation process.
- If this value exceeds 10^5 CFU/g there is potential emetic toxin presence.
- If growth is not present (i.e. lag time is longer than storage time), none of the spores germinate. If the spore concentration exceeds 10^5 spores/g there is a potential for diarrhoeal syndrome.

Because the logic is different and because it is not a change in production or consumer behaviour, this scenario is not included in Table 8.2.

8.2.2 Evaluation and ranking of scenarios according to impact

Because many assumptions remain in the model, our intent is not to quantify the absolute or numerical effect of a scenario, but rather to compare them relative to one another. To describe the relative effect of a scenario on the number of 'risky' packages, four magnitudes of effect are used:

- No effect (< 1%)
- Minor effect (1 - 20%)
- Moderate effect (20 - 80 %)
- Major effect (>80%).

Table 8.1: Difference in parameter values used in the scenarios and the parameter values used in the baseline scenario (chapter 7)

Scenario		Parameters changed		Value	Value	Reference[a]	
nr	Description	Name	Description	in baseline	in scenario	Table nr.	Page
1	Improved raw material selection	*Spec*	*B. cereus* specification for raw materials [log CFU/g]	4	3	7.2	173
2	Best-case hygiene at handling	*ContactHand*	*B. cereus* Contamination on contact and non-contact materials [log CFU/25cm^2]	RiskNormal(-0.20,1.21)	-4.89	7.3	175
		NonContactHand		RiskNormal(-1.59,1.49)	-7.15		
3	Worst-case hygiene at handling	*ContactHand*	*B. cereus* Contamination on contact and non-contact materials [log CFU/25cm^2]	RiskNormal(-0.19,1.21)	4.65		
		NonContactHand		RiskNormal(-1.59,1.49)	4.66		
4	Intermediate storage 15h-10°C	t_{wait}	Waiting time [hr]	RiskUniform(0,4)	15	7.5	179
		StT	storage temperature [°C]	RiskPert(7,20,65)	10		
5	Intermediate storage 8h-22°C	t_{wait}	Waiting time [hr]	RiskUniform(0,4)	8		
		StT	storage temperature [°C]	RiskPert(7,20,65)	22		
6	Intermediate storage 15min-37°C	t_{wait}	Waiting time [hr]	RiskUniform(0,4)	0.25		
		StT	storage temperature [°C]	RiskPert(7,20,65)	37		
7	Best-case hygiene at packaging and assembly	*ContactAssem*	*B. cereus* Contamination on contact and non-contact materials [log CFU/25cm^2]	RiskNormal(0.08,1.0)	-3.81	7.8	184
		NonContactAssem		RiskNormal(-0.67,1.34)	-5.73		

Continued on next page

Table 8.1 – continued from previous page

Scenario		Parameters changed		value	value	Ref[a]	
nr	Description	Name	Description	in baseline	in scenario	Table	Page
8	Worst-case hygiene at packaging and assembly	*ContactAssem* *NonContac-tAssem*	*B. cereus* Contamination on contact and non-contact materials [log CFU/25cm^2]	RiskNormal(0.08,1.0) RiskNormal(-0.67,1.34)	3.83 5.14	7.8	184
9	Pasteurisation at 87°C for 10min	HTT_{pasteur}	Pasteurisation temperature [°C]	90	87	7.9	187
10	Pasteurisation at 95°C for 5min	HTT_{pasteur} HT, t_{pasteur}	Pasteurisation temperature [°C] Pasteurisation time [min]	90 10	95 5	7.9	187
11	Shelf life one week shorter	*SL*	Shelf life [days]	28	21	7.11	189
12	No consumers with ‘no’-respect for the ‘use by’ date	in TTC[b]	Number of days margin taken on the ‘use by’ date	{0, 3, 7, 60}	{0, 3, 7}	7.11	189
13	No consumers with ‘no’- or ‘little’- respect for the ‘use by’ date	in TTC[b]	Number of days margin taken on the ‘use by’ date	{0, 3, 7, 60}	{0,3}		
14	Only consumers with ‘strict’-respect for the ‘use by’ date	in TTC[b]	Number of days margin taken on the ‘use by’ date	{0, 3, 7, 60}	{0}		
15	Only ’reasonable’ fridge temperatures	Truncation on T_7	Maximum temperature in the consumer fridge [°C]	17	8	7.10	188

Continued on next page

Table 8.1 – continued from previous page

Scenario		Parameters changed		value	value	Ref[a]	
nr	Description	Name	Description	in baseline	in scenario	Table	Page
16	Not all *B. cereus* strains can produce the emetic toxin	See section 8.2.1.9					

[a] in chapter 7, [b] see chapter 6

8.3 Results and discussion

An overview of the effect of the different scenarios on the output of the exposure assessment is given in Table 8.2. Output is expressed as the number of 'risky' packages (per million), prevalence (%) and *B. cereus* concentration (log CFU/g). If needed, more specific values are given in the text (e.g. output after a specific module in the QMEA).

Of the fifteen scenarios in which only one parameter was changed, only two had a major effect (>80%) on the exposure (8 and 15), four had a moderate effect (20-80%) on the exposure (3, 5, 7 and 11) and the other nine had only a minor effect (1-20%). The scenarios with a major effect were submitted to extra testing.

8.3.1 Scenarios with increased consumer exposure to *B. cereus*

8.3.1.1 Minor increase (↗)

The only scenario that caused a minor increase in the exposure was sc. 4: **intermediate storage for 15h at 10°C.** In this scenario, there were less batches with spore germination and hence there were less packages with cells and possible toxin formation compared to the baseline scenario (Table 8.2). The prevalence of *B. cereus* cells after waiting was 7.3% (±0.02%) compared to 30.1% (± 0.04%) in the baseline scenario . However, because none of the spores present germinated, the prevalence of spores was higher (by ± 5%) than in the baseline scenario. This resulted in an increased prevalence throughout the rest of the production process and the shelf life (± 3%). In addition, there was also a change to the spore and cell concentration after storage, the median spore concentration increased with 0.2 log CFU/g (-2.9 → -2.7). while the median cell concentration decreased with 0.2 log CFU/g (-2.1 → -2.3). The higher spore prevalence in the final product meant that there were more packages with the potential to develop high counts of *B. cereus* if conditions in the cold chain were favourable for growth.

8.3.1.2 Moderate increase (↑)

Three scenarios gave rise to a moderate increase in exposure: sc. 3 (Worst-case hygiene at handling), sc. 5 (intermediate storage for 8h at 22°C) and sc. 9 (pasteurisation for 10 min at 87°C).

Worst-case hygiene at the handling stage did not cause a notable change to the number of batches with possible toxin formation. However, it did result in a considerable increase in prevalence and *B. cereus* concentration. After thermal preparation, the prevalence was ±16% higher

than in the baseline scenario. After partitioning, the prevalence was ±40% higher (21.9% → 62.2%) and during shelf life the prevalence was still ±10% higher than in the baseline. The worst-case hygiene also affected the median concentration of the contaminated packages, which was 3.5 log CFU/g higher after handling compared to the baseline scenario (-1.6 → 1.9). The effect was also visible throughout the rest of the production process. After pasteurisation, the median concentration in the scenario was still 1.1 log CFU/g higher than in the baseline scenario.

Intermediate storage for 8h at 22°C caused a considerable increase (437 → 2391) in the number of batches with possible toxin formation (Table 8.2). The effect is opposite to that in scenario 4; the higher temperature means that much more batches will contain vegetative cells that can grow to concentrations of 10^5 cells/g during storage. These germinated spores lose their heat resistance and are inactivated during pasteurisation. Despite the large effect on toxin formation, the effect on prevalence or spore concentration is small. During the rest of the production and shelf life the prevalence, median spore concentration and even the 99^{th} percentile of the spore concentration are identical to the baseline scenario. Hence, there is almost no change in the number of packages that contain more than 10^5 CFU/g at the moment of consumption. However, the QMEA model only simulated one package per batch. In reality the number of batches will be lower (e.g 10^3 per year instead of 10^6 per year), but the number of packages from a single batch may be several thousand. So an increase in the number of batches with possible toxin presence should be avoided.

The last scenario to produce a moderate increase in exposure was a **milder pasteurisation treatment** (10 min at 87°C instead of 10 minutes at 90°C). Since the pasteurisation takes place after the intermediate storage, it did not affect the number of packages that may contain toxins. The 3°C difference only had a minimal effect on the spore concentration. Both median and 99^{th} percentile of spore concentration after pasteurisation did not differ from to the baseline scenario. However, the milder pasteurisation did result in a ±10% increase in prevalence during shelf life. Since the median and 99^{th} percentile of concentration did not change, this is probably caused by packages containing only a small number of spores.

8.3.1.3 Major increase (⇑)

There was only one scenario that brought about a major increase in exposure: **Worst-case hygiene during packaging and assembly**. The prevalence after assembly increased from an already high 87% to 100%, meaning that all packages contained at least 1 *B. cereus* spore. In addition the median *B. cereus* concentration increased from -1.4 to 3.2 log CFU/g, indicating a

considerable shift of the contamination distribution to the right. The 99th percentile of concentration showed a similar increase from 2.2 to 4.4 log CFU/g. The effect persisted after pasteurisation with prevalence being almost double that of the baseline scenario (48.6% → 90%) and the median concentration ±3.7 log CFU/g higher during shelf life. Apparently, worst-case hygiene drastically increased the prevalence and hence the probability for spores to end up in favourable conditions. If prevalence would be 1%, the probability of this one package being stored in a fridge with temperature abuse is rather small. However, if the prevalence is 100% then the probability of *B. cereus* growing equals the probability that a packages is stored under temperature abuse.

Because the effect of this scenario was very large, the scenario was subjected to further investigation. Two items were tested: (i) the effect of the parameters used in the module for recontamination during assembly and packaging (chapter 7, Section 7.2.2.7) and (ii) the effect of the assumption that all *B. cereus* entering the product are spores.

The first item was tested by performing a sensitivity analysis on the different parameters used in the model (See section 7.2.3 on page 171 for methodology and table 7.8 p. 184 for the different parameters). The scenario predicts that 2.4% of packages will contain more than 10^5 CFU/g or might contain emetic toxins. Figure 8.1 shows the difference between model outputs for the baseline scenario (blue bars) and the scenario 8 (red bars). This sensitivity analysis showed that changing model parameters could change this prediction into values between 1.3% and 3.5%. Certain model parameters have a considerable effect on the model output, especially in scenario 8 (e.g. time exposed to recontamination), but even in the best case (1.3%), this scenario is still the one that results in the highest exposure (of the sixteen scenarios tested).

The magnitude of this scenario may also be due do the assumption that all *B. cereus* entering the product are spores. In reality *B. cereus* entering the product from raw materials or the environment will be a mix of cells and spores. And if they are present as spores, they usually germinate fairly quickly. Although the rate of germination depends on the temperature (Rajkovic *et al.*, Accepted). If the recontamination contains more cells than spores, this step will have less effect on the exposure, because the cells will be inactivated during pasteurisation. To test the importance of this assumption, a simulation with an alternative assumption was run. In this simulation, it was assumed that only 0.1% of the *B. cereus* entering the package through recontamination at the assembly and packaging stage were spores. This reduced the exposure to 0.9% 'risk' packages (compared to 0.47% in the baseline scenario and 2.4% in the scenario). This shows that the assumption that all *B. cereus* are spores will considerably increase the effect of this scenario. But even with a much more lenient assumption, the exposure in this scenario is still double of the

baseline scenario. Hence, it is important to prevent recontamination via the environment or via the addition of untreated raw materials (e.g. grated cheese).

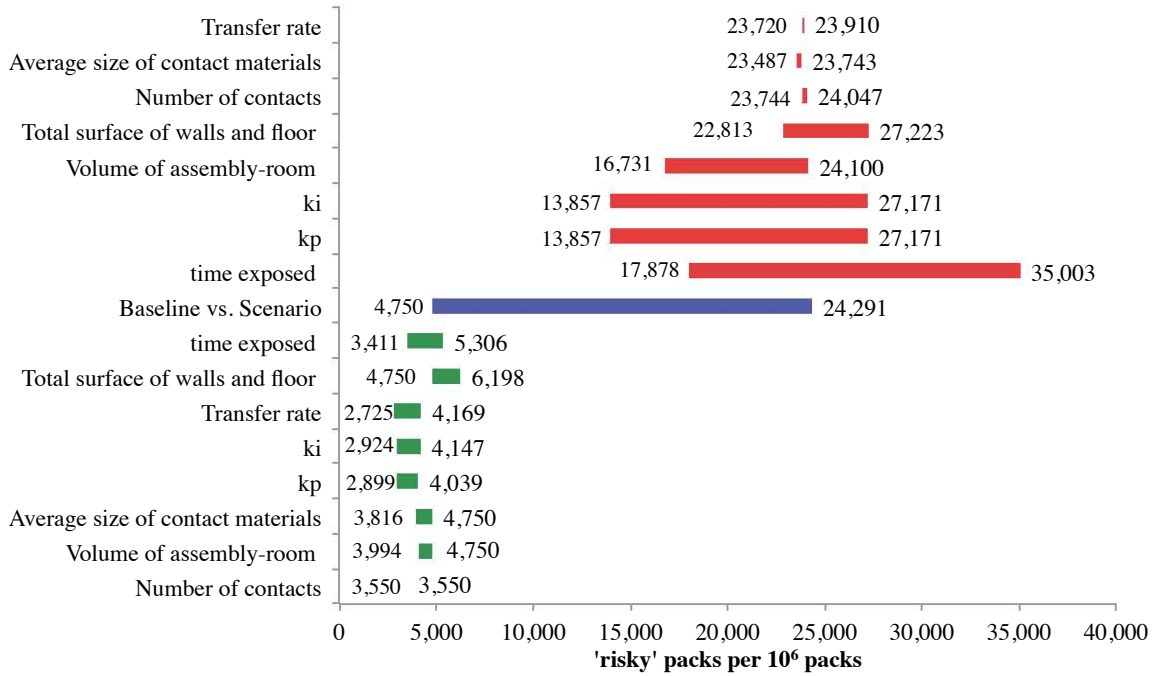


Figure 8.1: Tornado plot of the the parameters in module 7 (Table 7.8, p. 184) with their effect on the final model output. Bars show the difference in model output ('risky' packs per 10^6 packs) when the parameters in question is fixed (e.g. transfer rate), first at its 1^{st} percentile value (e.g. 0°C), and second at its 99^{th} percentile value (13°C), while all other parameters remain variable. Green bars are calculated using the baseline scenario (i.e. standard level of hygiene), red bars are calculated using scenario 8 (i.e. worst-case hygiene), the blue bar represents the difference between the baseline scenario and scenario 8

8.3.2 Scenarios with decreased consumer exposure to *B. cereus*

8.3.2.1 Minor decrease (↘)

Seven scenarios caused a minor decrease in exposure, namely 1, 2, 6, 10 and 12-14.

Scenario 1 **(Improved raw materials selection)** led to a considerable reduction (±75%) in the number of packages with possible toxins, but caused only a small change in the number of batches with elevated *B. cereus* concentrations during shelf life. At first glance, the scenario

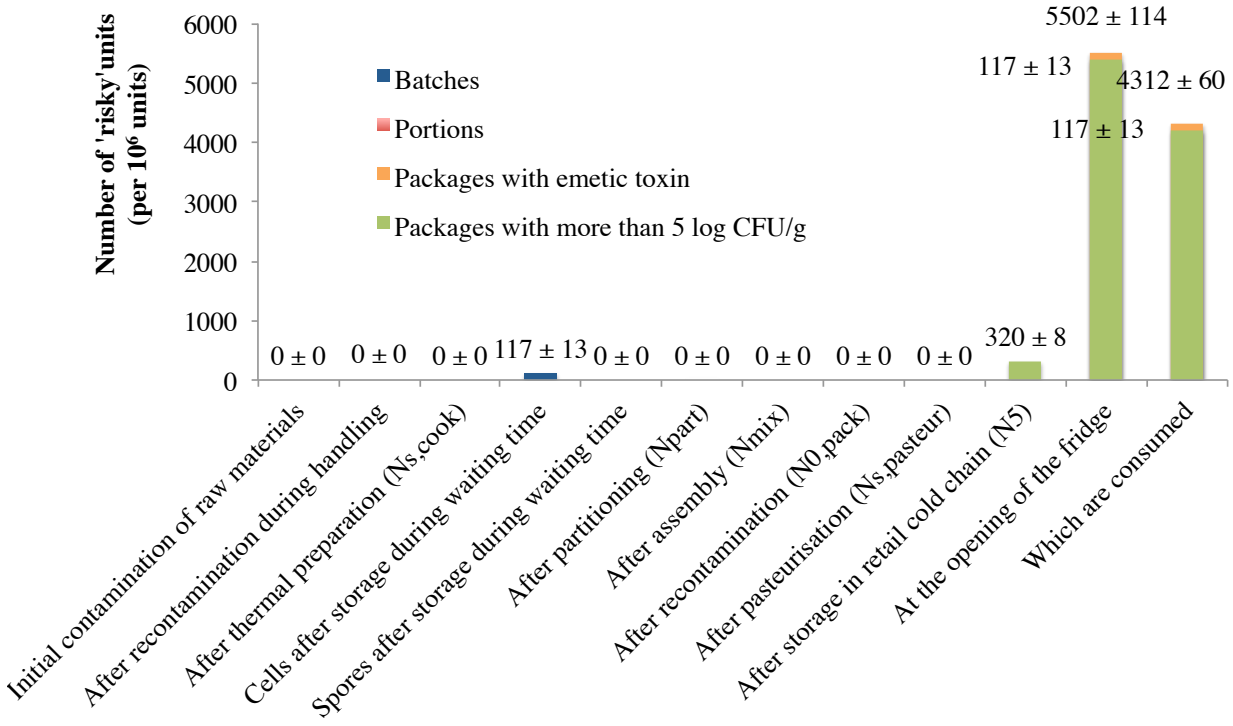


Figure 8.2: Scenario 1 (improved raw material selection). Number of units (i.e. batches, portions or packages) with a *B. cereus* concentration ≥ 10^5 CFU/g (for 10^6 iterations) throughout the REPFED production and shelf life. (± Standard deviation based on 3 simulations). Different colours represent different unit sizes.

seems to have a large effect (Figure 8.2), because during production there are no batches, portions or packages that contain more than 10^5 spores/g and only 117 batches contain enough *B. cereus* cells to allow toxin production. However, this effect is due to the truncation limit for raw material contamination which is set at 10^5CFU/g (compare to 10^6 CFU/g in the baseline scenario). In the baseline scenario, highly contaminated raw materials would show up above the threshold. However, in this scenario, the maximum concentration in a raw material group cannot exceed 10^5 spores/g. Because these groups are mixed together and because it is unlikely that two groups will be highly contaminated at the same time, the contamination is always lower than 10^5 spores/g. However, the improved raw material selection does not improve the overall raw material contamination. The median concentration (in the contaminated packs) remains unchanged compared to the baseline scenario, and there is only a small decrease in the 99th percentile of the *B. cereus* concentration during production (± 0.5 log CFU/g).

Scenario 2 (**Best-case hygiene during handling of raw materials**), is the scenario with the

smallest effect of all the tested scenarios. It has no notable effect on the number of batches with possible toxin presence and only a minimal effect on the number of packs with more than 10^5CFU/g. Apparently, excellent hygiene at this level of production does not necessarily translate into reduced risk.

Scenario 6 **(intermediate storage for 15 min at 37°C)** has a considerable impact on the number of batches containing toxins and also slightly reduces the number of packs with high counts during shelf life. The reason is similar as for scenario 4 (section 8.3.1.1). In scenario 4 *B. cereus* spores did not germinate, and hence no toxins were produced, but the spores remained present. In the current scenario, most spores germinate but dot not have sufficient time to grow to high concentrations.

Scenario 10 shows that **pasteurising at 95°C for 5min**, and thus at higher temperature for a shorter time, is more effective at prolonging the lag phase during shelf life. It also caused a minor reduction in *B. cereus* prevalence in the final product (46.8%→42.7%). However, there was no change in the median *B. cereus* contamination and very little change in the 99th percentile of *B. cereus* contamination (0.2 log CFU/g).

Scenarios 12, 13 and 14 each simulated different degrees of respect for the 'use by' date. They illustrate that consumers who do not (completely) respect the 'use by' date have a higher exposure. The more 'strict' consumers are in their respect for the 'use by' date, the lower the exposure. However, this effect is considerably smaller than that of the fridge temperature (section 8.3.2.3)

8.3.2.2 Moderate decrease (↓)

Two scenarios gave a moderate decrease in exposure: scenario 7 - best-case hygiene at packaging and assembly - and scenario 11 - shortening the shelf life by one week.

While worst-case hygiene at packaging level caused a major increase of the exposure, **best-case hygiene** (scenario 7) only caused a moderate decrease. Prevalence after recontamination was down by 60% and during shelf life it was nearly 30% lower (46.8% → 19.8%), but the median concentration just after packaging did not change compared to the baseline scenario. This is because, even in the baseline scenario, recontamination during packaging only adds a low number of spores to a package. Therefore, the good hygiene is most visible in the prevalence and not in the concentration.

Shortening the shelf life by one week (25%) caused the second largest decrease in exposure (4750 'risky' packs per 10^6 → 3731 'risky' packs per 10^6), despite the fact that the majority of

the products are eaten within the first week. In the baseline scenario (shelf life of 28 days) ca. 3.8% of the products is discarded instead of consumed, in the scenario with shortened shelf life, this is 5.9%. This larger portions of discard products, may account for some of the decreased exposure. In addition, a shorter 'use by' date will shorten the 'sell-by' date and hence products will spend less time in the retail.

8.3.2.3 Major decrease (⇓)

Only one scenario caused a major decrease in exposure: scenario 15 - **only 'reasonable' fridge temperatures**. Although the prevalence was unchanged, the number of packs with more than 10^5 spores or cells during shelf life decreased by almost 90% (4313→492). In addition the 99th percentile of the *B. cereus* concentration decreased by 2 log CFU/g, while the median concentration did not change. This scenario illustrates that consumer behaviour is a vital aspect in reducing the exposure. Nauta (2001), reported similar results in his exposure assessment for *B. cereus* in a broccoli bases REPFEDs. If the temperature in consumer fridges was fixed at 7°C, the % of packages with more than 10^5 CFU/g decreased considerably. Garrido *et al.* (2010) reported similar results for a scenario-analysis for listeriosis. Lowering the consumer refrigerator temperature was the most effective way to reduce listeriosis.

Like for scenario 8, it was deemed appropriate to further investigate this scenario given its considerable impact on the exposure. The effect of two parameters was tested on the effect of this scenario: (i) product pH and (ii) % of *B. cereus* strains able to grow at ≤8°C.

The pH was tested, because it became clear during the modelling (chapter 4 and 5), that pH and heat treatment had a synergistic effect. A heat treatment was more effective in prolonging the lag time if pH was suboptimal, even at a moderate pH decrease. To determine if this changed the outcome of the scenario, both the scenario and the baseline model were rerun with pH 6.3 instead of 5.95. Although, this may seem only a small increase, it was observed in chapter 5, that above pH 6.2 the heat treatment had no significant effect on the lag. When the pH-value was changed to 6.3, the baseline model predicted 1944 (1.04%) of 'risky' packages and scenario 15 predicted 10383 (0.19%). The ratio between both (1944/10383 = 0.2) is almost identical to that found at pH 5.95 (929/4750=0.19). This means that at higher pH, the effect of this scenario is still valid. It also stresses the large effect of pH on the exposure.

In the baseline model, it was assumed that only 6.2% of *B. cereus* strains could grow at temperatures ≤8 °C (Table 7.11 p. 189). To test whether this assumption changed the relevance of this scenario, the scenario and the baseline model were rerun with the assumption that 30% of the

B. cereus strains could grow at temperature ≤8°C. Changing this assumption did not change the impact of this scenario compared to the baseline model. With the new assumption, the baseline model predicted 0.62% 'risk' packages (0.48% with standard assumption) and scenario 15 predicted 0.097% 'risky' packages (0.093% with standard assumption). This means that changing the assumption had more effect on the baseline model, than on this scenario. It also means that the importance of consumer fridge temperature still stands, even if the fraction of psychrotrophic strains would be higher than the assumed 6.2%.

8.3.3 Scenario 16

In the baseline scenario it is assumed that all *B. cereus* strains (100%) can produce the emetic toxin. In this scenario the effect of a different assumption was tested: that only 3.2% of the *B. cereus* strains can produce the emetic toxin. The number of batches with toxin presence during intermediate storage decreased drastically from 437 (±27) to 14 (±2). The number of packages at the end of shelf life was split in two: 1324 (±44) contained more than 10^5 cells/g of a strain that could form the emetic toxin; Only 6 (±2) contained high spore counts (≥10^5 spores/g). This shows that our original assumption has a considerable impact on the exposure assessment. However, even the output of this scenario may still be an overestimation. In the scenario, the assumption was used that all the *B. cereus* spores present could be from different strains, which is unlikely (Rajkovic *et al.*, Accepted).

Table 8.2: Summary output of the different scenario's. 'Risky' packs are packs with either $\geq 10^5$ CFU/g at the moment of consumption or originating from batches that contained $\geq 10^5$ cells/g during intermediate storage. The arrows illustrate the relative effect of the scenario on the total number of 'risky' packages compared to the baseline scenario. 'risky packages are packages with $\geq 10^5$CFU/g or with toxin formation during production. "=" No change; ↘ or ↗: Minor decrease or increase (1- 20%) ; ↓ or ↑: Moderate decrease or increase (20 - 80%; ⇓ or ⇑; Major decrease or increase (>80%).

Scenario		Number[a] of packs with:		'Risky' packs[a]	Prevalence	Concentration[b]		Relative
nr	Description	$\geq 10^5$ CFU/g	toxins			Median	99th-%	exposure
0	Baseline (Chapter 7)	4313 ± 49	437 ± 27	4750 ± 67	46.8% ± 0.02	-1.7 ± 0	4.7 ± 0.049	=
1	Improved raw material selection	4195 ± 60	117 ± 13	4312 ± 73	46.5% ± 0.03	-1.7 ± 0	4.6 ± 0.068	↘
2	Best-case hygiene at handling	4195 ± 68	444 ± 12	4638 ± 61	46.6% ± 0.03	-1.7 ± 0	4.6 ± 0.05	↘
3	Worst-case hygiene at handling	6564 ± 124	426 ± 20	6990 ± 113	58.6% ± 0	-0.5 ± 0	5.4 ± 0.081	↑
4	Intermediate storage 15h-10°C	4858 ± 38	48 ± 4	4905 ± 42	48.1% ± 0.01	-1.6 ± 0	5 ± 0.026	↗
5	Intermediate storage 8h-22°C	4197 ± 43	2391 ± 42	6588 ± 40	46.7% ± 0.04	-1.7 ± 0	4.6 ± 0.042	↑
6	Intermediate storage 15min-37°C	4162 ± 29	32 ± 8	4195 ± 22	46.1% ± 0.02	-1.7 ± 0	4.6 ± 0.021	↘
7	Best-case hygiene at packaging	1597 ± 77	438 ± 47	2035 ± 124	19.8% ± 0.02	-1.4 ± 0	4.4 ± 0.101	↓
8	Worst-case hygiene at packaging	23848 ± 25	443 ± 10	24291 ± 26	87.1% ± 0.02	2.1 ± 0.002	8.4 ± 0.013	⇑
9	Pasteurisation 87°C 10min	5829 ± 24	448 ± 38	6277 ± 16	57% ± 0.02	-1.6 ± 0.001	5.1 ± 0.008	↑
10	Pasteurisation 95°C 5min	3668 ± 56	427 ± 34	4095 ± 76	42.7% ± 0.03	-1.7 ± 0	4.5 ± 0.05	↘
11	Shelf life one week shorter	3303 ± 22	428 ± 10	3731 ± 31	45.7% ± 0.04	-1.7 ± 0	4.1 ± 0.009	↓
12	No consumers with 'no' respect for the 'use by' date	4139 ± 8	435 ± 18	4574 ± 26	46.7% ± 0.04	-1.7 ± 0	4.6 ± 0.01	↘
13	No consumers with 'no' or 'little' respect for the 'use by' date	4081 ± 64	438 ± 25	4519 ± 43	46.7% ± 0.05	-1.7 ± 0	4.5 ± 0.049	↘
14	Only consumers with 'strict' respect for the 'use by' date	3816 ± 55	427 ± 19	4243 ± 58	45.7% ± 0.01	-1.7 ± 0	4.4 ± 0.028	↘
15	Only 'reasonable' fridge temperatures	492 ± 15	437 ± 16	929 ± 7	46.8% ± 0.02	-1.7 ± 0	2.6 ± 0.017	⇓

[a] per 10^6 packs; [b] *B. cereus* in the contaminated packs, expressed in log CFU/g

8.4 Conclusions

The output of scenario 16 shows that is important not to interpret the QMEA output as an absolute estimate, but rather to use the scenarios to assess the relative impact of processing steps or consumer behaviour. Ten scenarios caused a decrease in exposure, six caused an increase in exposure. Eight scenarios had only a minor effect on the exposure, five had a moderate effect, and only two had a major effect: (i) worst-case hygiene during packaging and assembly and (ii) only 'reasonable' fridge temperatures.

Four conclusions can be made for the REPFED producers. They concern pasteurisation, intermediate storage and hygiene. First, **pasteurisation** at higher temperature for shorter times is preferable to lower temperature for longer times. Second, **intermediate storage** should be kept as short as possible to prevent germination, outgrowth and toxin formation by *B. cereus*. The temperature during intermediate storage is of less relevance, because it is difficult to cool the large volumes (400kg) to temperatures low enough to prevent growth of *B. cereus*. Third, reducing the **shelf life** will reduce the exposure but will cause an increase in the percentage of products that is discarded instead of consumed. A cost-benefit analysis should be made between the 'safety-first' approach (i.e. shorter shelf life) and sustainability (reducing food waste). Finally, **hygiene** during processing needs attention, although perfect hygiene during handling of raw materials may not reduce exposure, low hygiene at this stage will cause an increased exposure. This effect is even more pronounced at the assembly and packaging process. Bad hygiene at this stage will cause a drastic increase in exposure ($\times$5, 4,750$\rightarrow$24,291 'risky' packages per million); excellent hygiene will reduce the exposure ($\times$0.5, 4,750$\rightarrow$2,035 'risky' packages per million).

Producers should also be aware of the effect of a longer shelf life. It is our understanding, that the **long shelf lives** given to REPFEDs are often demanded by either the marketing department or by the retail (personal communications). Producers – or the retail? – should take into account that 90% of products are consumed within a week after consumption and that shortening the shelf life from 4 weeks to 3 weeks, reduces the exposure with approximately 20%. The fact that shelf lives longer than 21 days have only a minor added-value for the consumer, but do cause significant additional exposure, has to be considered.

Competent authorities must take into account that demanding a certain pasteurisation treatment is not sufficient; selection of raw materials and hygiene must also be controlled by the company (e.g. sampling plans?). In addition to producers, consumers also have a responsibility because temperature abuse by the consumer is the single most important factor in exposure to *B. cereus*

from REPFEDs. Education might improve consumer awareness about the effect of their behaviour. However, multiple studies have shown that it is not easy to change consumer habits. It is a process that requires collaboration between food microbiologists/risk assessors and social scientist (Fisher *et al.*, 2005).

Chapter 9

A Quantitative Microbiological Exposure Assessment of *B. cereus* in REPFEDs: Part 3 - iso-risk

Summary

Reducing the heat treatment intensity (i.e. time/temperature) in the production of REPFEDs, can further improve product quality, both nutritional and sensorial. However, when reducing the heat treatment, it is important to introduce other hurdles in order not to compromise consumer food safety. The developed predictive models for heat treated *B. cereus* spores (chapters 4 and 5) and the exposure assessment for *B. cereus* (chapter 7) demonstrated that pH, heat treatment and shelf life all affect the consumer exposure to *B. cereus* spores and toxins from REPFEDs. To set boundaries in which product and process characteristics can be changed without increasing consumer exposure, a set of iso-risk curves is presented in this chapter. Each combination of parameters (i.e. shelf life, pH and pasteurisation time and temperature) on these curves will result in a consumer exposure to *B. cereus* equal to the baseline exposure (±0.48% or 4750 packages per million). These iso-risk curves allow producers to choose feasible combinations of heat treatment, pH and shelf life, which predict the same exposure, but also live up to the consumer's quality expectations. However, combinations selected based on the iso-risk curves, will still have to be validated in an actual food product using challenge tests and durability tests.

9.1 Introduction

During the development of the predictive models for *B. cereus* (chapter 4 and 5), the effect of pH, a_w and pasteurisation on the lag time of *B. cereus* was determined. In the quantitative microbiological exposure assessment (chapter 7) and scenario analysis (chapter 8) the effect of shelf life and pasteurisation on the consumer exposure to *B. cereus* was investigated. In this chapter the two approaches are combined. The goal is to illustrate that the QMEA can be used to determine sets of iso-risk curves. An iso-risk curve is a graphical representation of combinations of pH, heat treatment intensity and shelf life that all lead to the same risk. Each point on these curves, i.e. each combination of parameters, will lead to the same level of exposure as the baseline model: ± 0.48% 'risky' packages. In the QMEA, a package was considered 'risky' for *B. cereus*, based on two possible conditions. First, if it contains 10^5 CFU/g *B. cereus* or more during shelf life. Second, if during production, toxin formation was possible in the batch from which the product originated. The approach used for the iso-risk curves is similar to the 'Degree of Protection' (DoP) approach for *C. botulinum* (see section 1.3.5, p.25) (Lund, 1993; Membré *et al.*, 2009; Membré, 2009).

The iso-risk curves have two applications, one in product innovation and one in operational support (Membré, 2009). For the first application, the iso-risk curves serve as a set of guidelines

for product development. For example, if a REPFED producer wants to decrease pasteurisation intensity, the iso-risk curves can be used to determine with how many days the shelf life should be reduced, or what is the required pH-shift, to result in the **same exposure** for consumers at the time of consumption. In the second application the iso-risk curves can be used as a form of **risk-boundaries** for a production process (e.g. in a HACCP plan, to defining limits for a critical control point). In this approach, the producer chooses a maximum tolerable risk/exposure and subsequently determines the heat treatment needed or the maximum possible shelf life, given the pH and a_w of the product. The curves presented all use the current exposure as maximum tolerable exposure (i.e. 0.48%). Interpreting the iso-risk curves as risk boundaries is similar to the growth/ no-growth models (chapter 4). Combinations (of pH, shelf life and pasteurisation) on one side of the curve will have a higher exposure, combinations of the other side of the curve will have a lower exposure.

Four different iso-risk curves are presented for *B. cereus* to demonstrate the first application of these curves, namely to determine the possibilities for modifying the product/process parameters without changing the consumer exposure. The second application of the iso-risk curves will be presented for both *B. cereus* and *C. botulinum*, because both are spore forming pathogens of concern in REPFEDs (Carlin *et al.*, 2000a). A comparison is made between the risk-boundaries (i.e. the iso-risk curves) for psychrotrophic *B. cereus* (based on the results of the QMEA in chapter 7) and the iso-probability curves for psychrotrophic *C. botulinum* presented by Membré *et al.* (2009) and Membré (2009). A comparison of the boundaries for both microorganisms will determine if there is a conflict between the exposure to *B. cereus* and *C. botulinum*. This comparison checks if changing the parameters (pH, shelf life, etc.), based on keeping the same risk for *B. cereus*, does not increase the risk for *C. botulinum*. It is necessary to verify that new combinations or new risk-boundaries still provide (at least) the same degree of protection for *C. botulinum*.

It is important to note that the curves in this chapter are based on the output of the QMEA in chapter 7, which uses the predictive model from chapter 5. This predictive model was based on experimental data. The experiments were done for a combination of heat treatment time (1-38 min), temperature (85, 90 or 95°C), pH (5.2 - 6.4), a_w (0.973-0.995) and storage temperature (8-30°C). The iso-risk curves include values that are outside these limits, i.e. that are extrapolated beyond the experimental limits. Although extrapolation is fairly easy from a mathematical point of view, it is always necessary to consider if the extrapolated predictions are still realistic. For the prediction of the QMEA, this implies that predictions with pasteurisation times shorter than 1 minute or longer than 38 minutes or pasteurisation temperatures lower than 85°C, or higher

than 95°C are to be critically evaluated. For illustrative purposes, the figures that are presented in this chapter include extrapolated values for heat treatment temperature and time. When using these curves for product innovation or for operational support, the prediction should not be extrapolated.

9.2 Methodology

Four iso-risk curves were constructed using the QMEA from chapter 7. For illustrative purposes, the iso-risk curves also include extrapolated value (i.e. values beyond the experimental limits of the lag model from chapter 5).

1. The required heat treatment **time** as a function of **pH** (5.4→6.4), for a given shelf life (21, 28 and 35 days) and heat treatment temperature (85, 90 and 95°C).

2. The required heat treatment **temperature** as a function of product **pH** (5.4 → 6.4), for a given combination shelf life (21, 28 and 35 days) and heat treatment time (10 min)

3. The maximum **shelf life** as a function of heat treatment **temperature** (85 → 95°C), for a given combination of product pH (5.7, 6.0 and 6.3) and heat treatment time (10 min).

4. The required heat treatment **time** as a function of **shelf life** (16→45 days) for a given combination of product pH (5.7, 6.0 and 6.3), heat treatment temperature (85, 90 and 95 °C).

The product a_w was set to 0.99 for all simulations, because most REPFEDs have a high a_w (Figure 9.1). A fixed a_w was chosen because it was found that a_w has limited influence on the lag time (Chapter 5) and because REPFED producers reported that the a_w of a product was difficult to change. For all other variables, the same values or distribution were used as in the original QMEA model (Chapter 7). For example, the temperature in a consumer refrigerator was a normal distribution with mean 6.7°C, standard deviation 2.8°C and truncated at 17°C (Table 7.10, p.188).

The different iso-risk curves in this chapter were calculated using a "directed trial-and-error method". As an example for figure 9.3 this meant that for each combination of pH and shelf life, the exposure was calculated for two heat treatment temperatures. These first two values were selected based on previous experience with the QMEA model. The output of these two simulations was then used to determine the 'correct' heat treatment temperature via simple regression. A value was deemed to yield the same exposure if the was equal to 4,750 (± 67) 'risky' packs

per 10_6 packs. The value found via regression was then verified in a third run. If the value was not confirmed, a new regression was performed using the output of the three simulations. This process was repeated until an 'equal' exposure was returned by the model. This typically took 4-5 simulation.

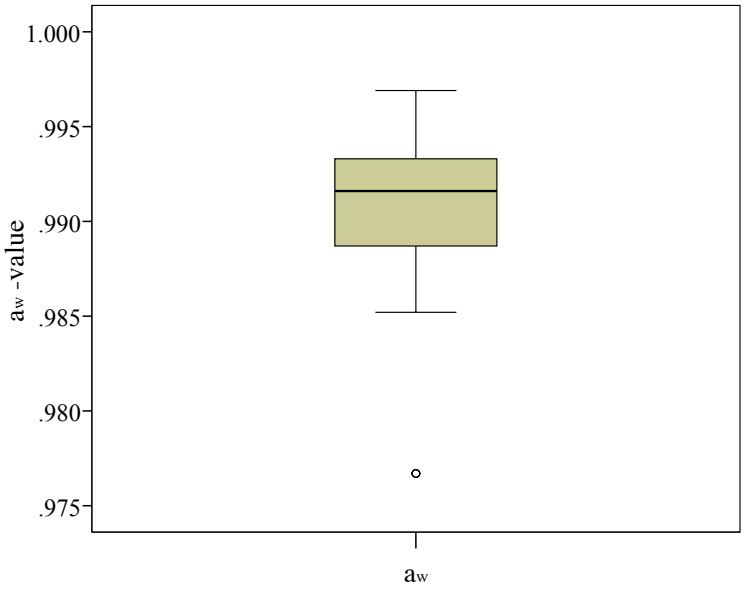


Figure 9.1: Box plot of the water activities of 30 REPFEDs, sampled for the assessment in chapter 2.

9.3 Results and discussion

9.3.1 Heat treatment time as a function of product pH

Figure 9.2 shows the heat treatment time required to obtain the same exposure, compared to the baseline model (i.e. 0.48%), as a function of pH for different heat treatment temperatures and shelf lives. It shows that the effect of pH and heat treatment temperature is larger than the effect of shelf life. For example, consider a reference product with a shelf life of 28 days, a heat treatment temperature of 90°C and pH 6.0. This product requires a heat treatment of 12 minutes. The same product with pH 6.2 will require a 17.8 minute heat treatment (Δ=5.8 min). If the shelf life of the reference product is increased to 35 days, the required heat treatment increases to 13.7 min (Δ=1.7 min). Lowering the heat treatment temperature of the reference product to 85°C, increases the required heat treatment time to 26.4 min (Δ=14.4 min). Increasing heat treatment temperature has a comparable but opposite effect (Heating time = 4.65 min, Δ=7.35 min).

At pH-values of 5.6 and below, the required heat treatment time is very short. At pH 5.6 and a shelf life of 35 days, a heat treatment of 1.5 minutes at 90°C is sufficient to limit consumer exposure to an equivalent level as the baseline scenario. At pH 5.4 and shelf life 35 days, a heat treatment of 0.03 minutes at 85°C is sufficient. It is important to note that this heating time is in the extrapolated area of the model (<1 min) and therefore should be interpreted with due attention. Additionally, the pasteurisation of REPFEDs should not only prevent growth of *B. cereus*, but should also inactivate vegetative pathogenic and spoilage microorganisms. With a heat treatment of 0.03 minutes (1.8 sec) it is unlikely that these are inactivated (Gaze, 2006). This will cause the product to spoil or to become a risk with respect to other pathogenic microorganisms. At the other end of the pH scale, there is a sharp increase of the heat treatment time required if the pH is higher than 6.2. This is a direct consequence of the parameterisation of the lag model. In the model, the optimal pH (pH_{opt} of *B. cereus* is 6.23 (Table 5.4, p.119)). Once the pH is higher than pH_{opt}, both pH and heat treatment no longer extend the lag time (Equations 5.3 and 5.5, p.112). Therefore, to achieve the same level of exposure, more inactivation is required and the heat treatment time increases significantly in product with pH higher than 6.23.

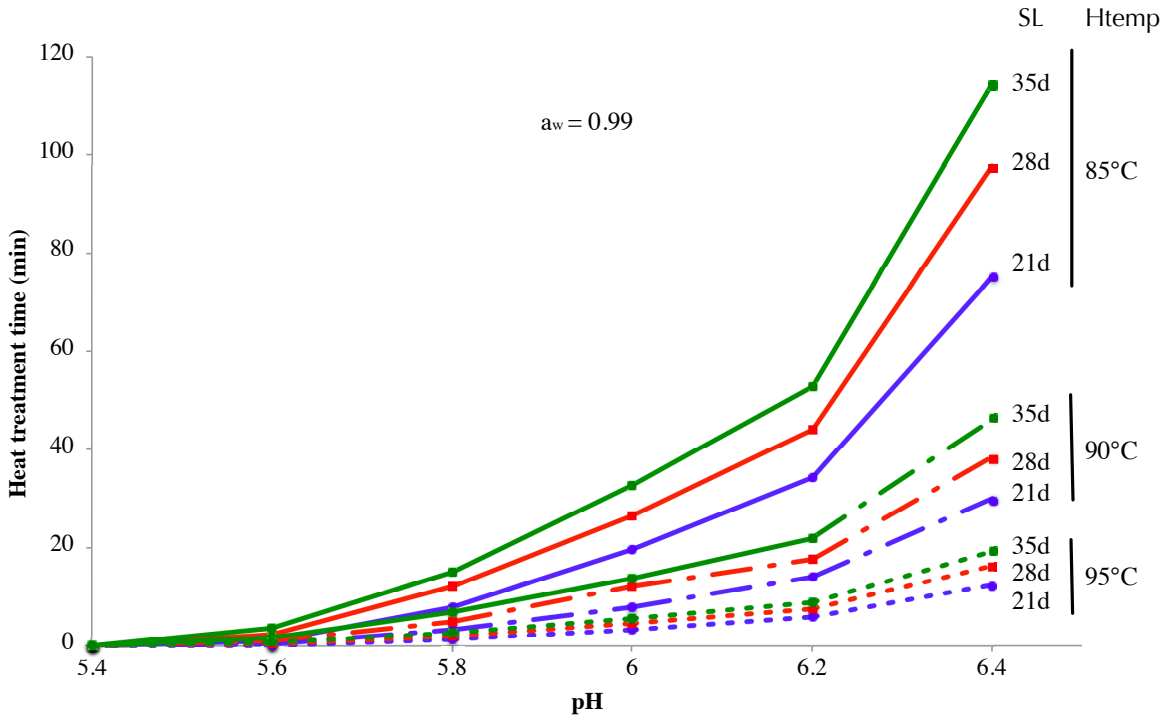


Figure 9.2: Iso-risk curves for different shelf lives (SL) and heat treatment temperature (Htemp) combinations. Heat treatment time needed (y-axis) to obtain the same % of 'risky packs' as in the baseline scenario, for a product with a given pH (x-axis) (a_w: 0.99).

9.3.2 Heat treatment temperature as a function of product pH

Figure 9.3 demonstrates the heat treatment temperature required to obtain a certain shelf life (21, 28 or 35 days) as a function of pH with a fixed heat treatment time (10 min). This iso-risk curve confirms that the effect of pH is larger than the effect of a change in shelf life. A pH difference of ± 0.2 requires a temperature change of ± 3°C, a shelf life difference of 7 days requires a temperature change of ± 1°C. There is one exception, at pH 5.6 the difference between a shelf life of 21 and 28 days is considerably larger than at other pH values. This sudden decrease in the required heat treatment temperature is a result of the parameterisation of the predictive model; i.e. below 85 °C, the heat treatment no longer affects the lag time because HT_{opt} in equation 5.10 was fixed at 85°C. At pH 5.6, the heat treatment temperatures for the two lowest shelf life (21 and 28 days) are 77.5 and 84.1°C respectively. For a shelf life of 35 days, the required heat treatment temperature is 85°C. This means that for 21 and 28 days, the heat treatment will cause inactivation, but will not increase the lag time. For a shelf life of 35 days, the heat treatment will prolong lag time. Hence, the graph appears misleading, with 28 and 35 days close together and 21 much lower.

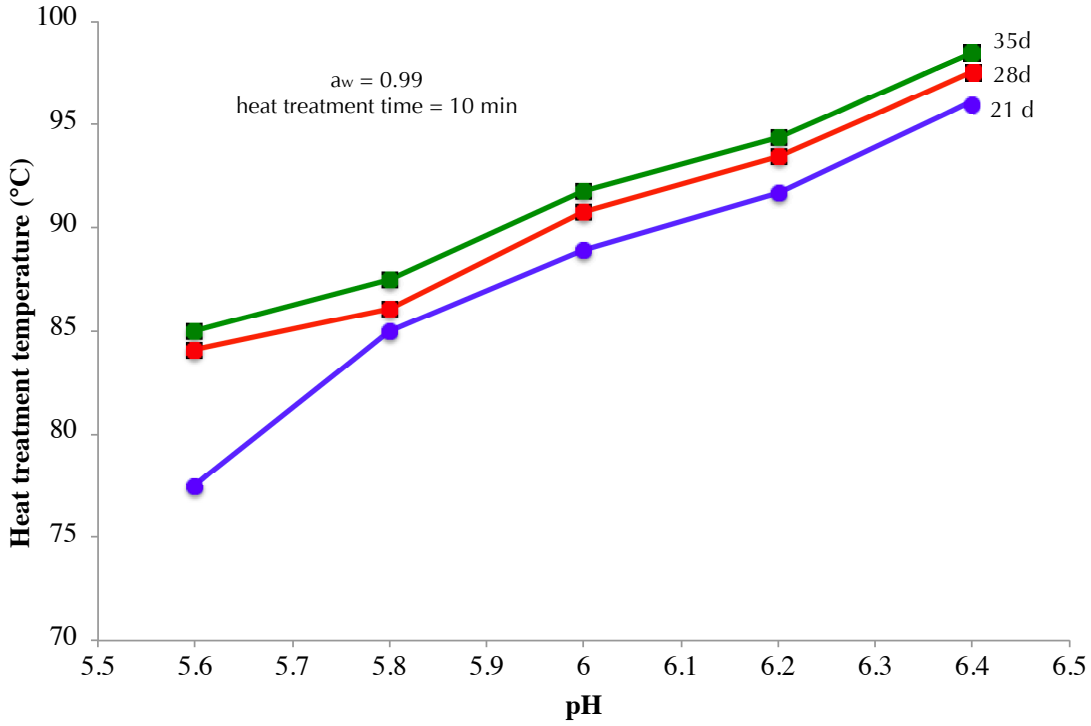


Figure 9.3: Iso-risk curves for different shelf lives, heat treatment temperature needed (y-axis) to obtain the same % of 'risky packs' as in the baseline scenario, for a certain product pH (x-axis) (a_w =0.99, heat treatment time =10 min).

It is important to note that while pH has a considerable impact on the lag time of *B. cereus* and therefore also on the exposure, lowering product pH is not a straightforward task with respect to sensorial changes. In addition, it is difficult to predict the pH of a product consisting of multiple components, each with different pH value (e.g. a tomato and béchamel sauce).

9.3.3 Maximum shelf life as a function of heat treatment temperature

Figure 9.4 illustrates the maximum shelf life (days) as a function of heat treatment temperature at different product pH-values (a_w 0.99, heating time 10 min). The graph demonstrates that at mildly acidic pH (5.7), a mild heat treatment (i.e. 10 minutes at 84 - 88°C) is sufficient to assure relative long shelf lives (30 to >90 days), while at higher pH (6.3), even intense heat treatments (98°C, 10 min) can hardly achieve long shelf lives (max. 35 days). The curve at pH 6.0 (green) also shows that for a heat treatment temperature increase from 85°C to 90 °C the maximum shelf life only increases with ten days (15→25 days), while an increase in heat treatment temperature from 90°C to 95°C results in a shelf life increase of 55 days (25→80 days).

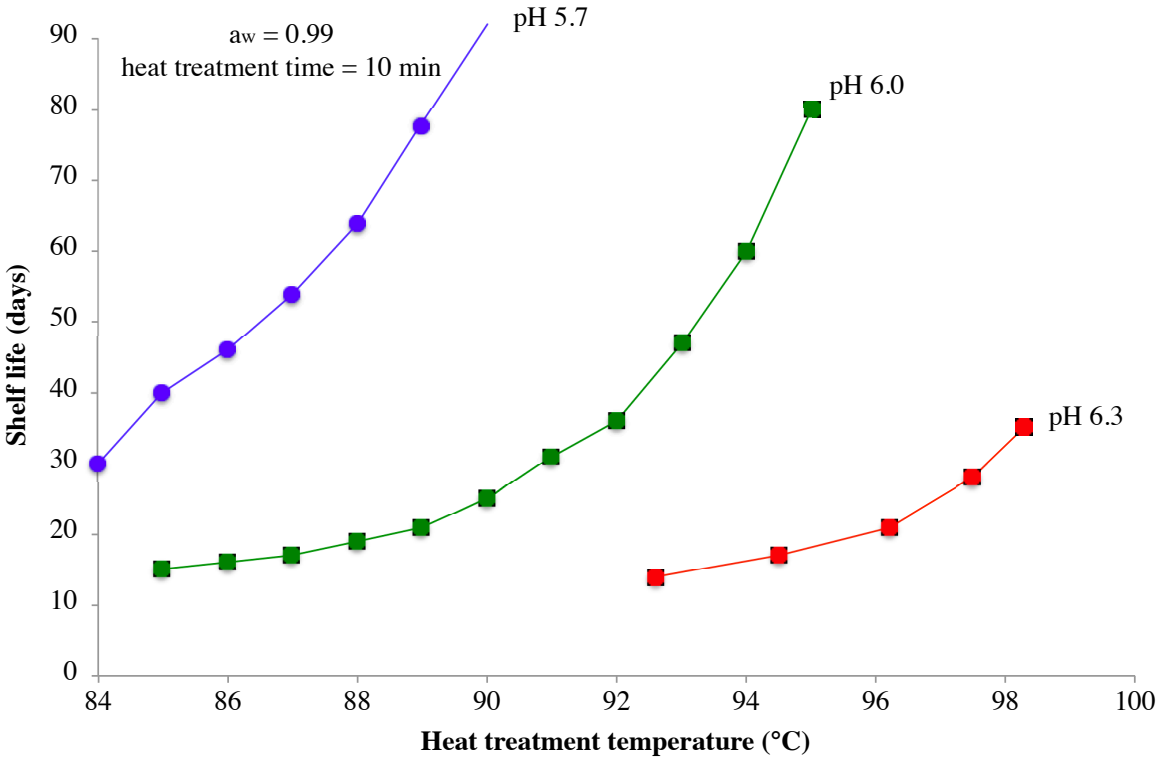
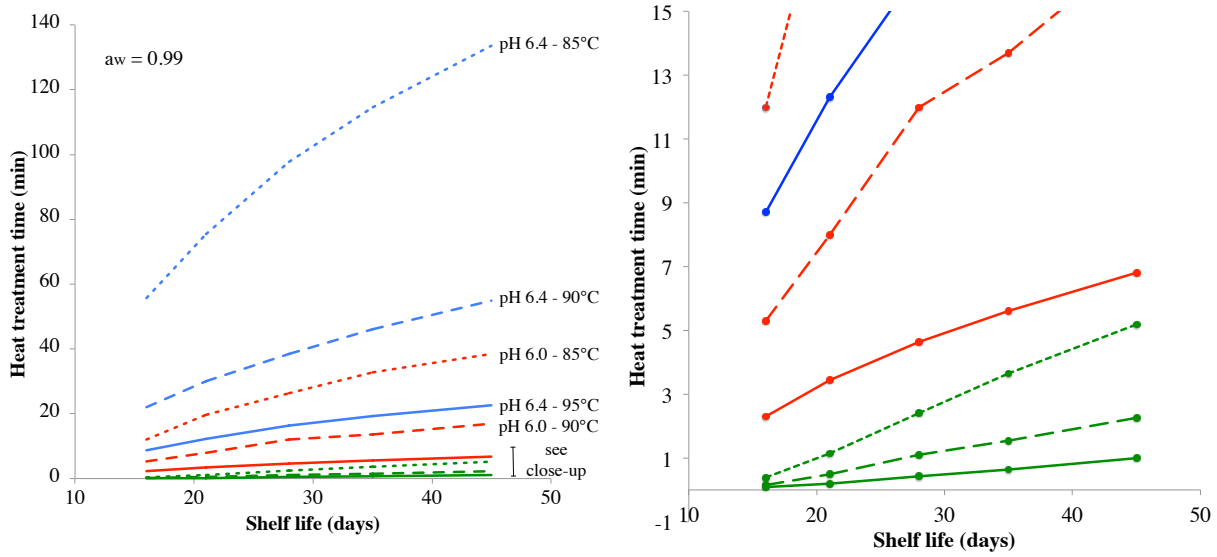


Figure 9.4: Iso-risk curves for different pH-values (blue: 5.7, green: 6.0 and red: 6.3), maximum shelf life as a function of heat treatment temperature to obtain the same % of 'risky packs' as in the baseline scenario (a_w:0.99, heat treatment time: 10min).

9.3.4 Maximum shelf life as a function of heat treatment time

The iso-risk curves in figure 9.5 shows the heat treatment time required to obtain a certain shelf life. It is clear that at higher pH values (i.e. pH 6.4), the required heat treatment time is considerably longer, even at heat treatment temperature of 90 or 95°C. The curves also demonstrate the synergistic effects of pH and heat treatment temperature. At low pH and high temperature (pH 5.6, 95°C), a shelf life of 45 days requires a heat treatment of 1 minute. The same shelf life at pH 6.4, requires 22 minutes at 95°C. At pH 5.6, it requires 5 minutes at 85°C. However, at pH 6.4 and 85°C, it requires no less than 133.5 min. The difference in heat treatment time was so large, that a second (close-up) graph was needed to clearly represent the values at low pH and high temperature.



(a)

(b) Close-up: Heating time 0-15 min

Figure 9.5: (a) Iso-risk curves for different combination of pH (blue: 6.4, red: 6.0 and green: 5.6) and heat treatment temperature (— 95°C,– – – 90°C, - - - 85°C). Maximum shelf life as a function of heat treatment time to obtain the same % of 'risky' packs as in the baseline scenario (a_w: 0.99). (b) close-up view of figure a for heat treatment times below 15 minutes.

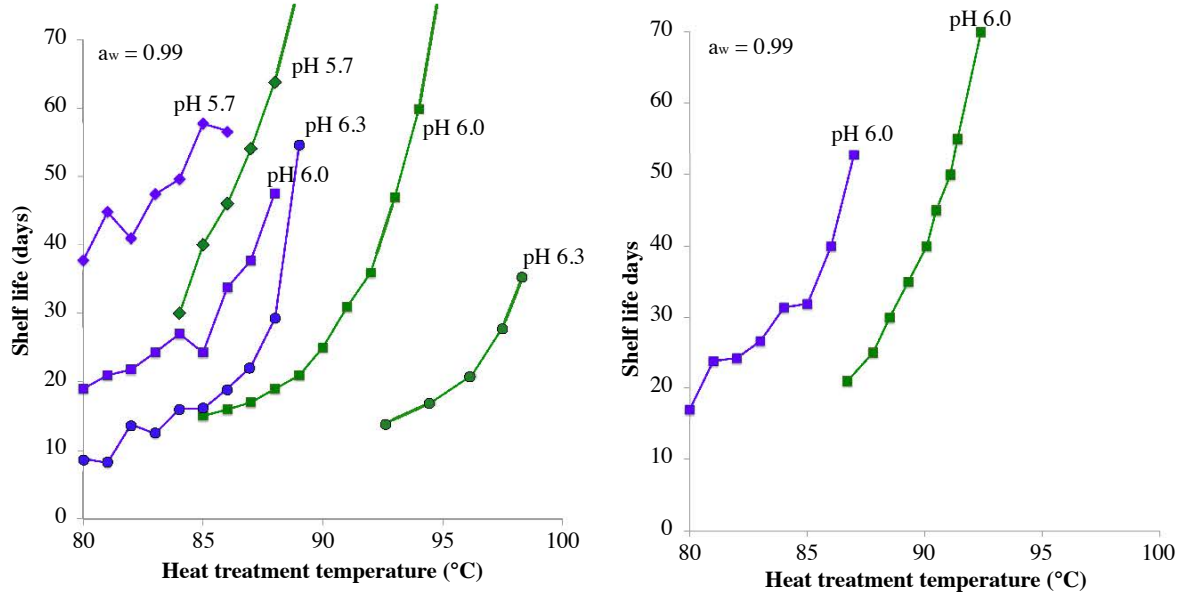
9.4 Risk-boundaries for *B. cereus* vs. *C. botulinum*

Both *B. cereus* and *C. botulinum* are organisms of concern in REPFEDs (Carlin *et al.*, 2000a). An alternative application of the iso-risk curves is to determine the 'risk-boundaries'. In this section the boundaries of *B. cereus* and *C. botulinum* are compared, based on the results presented for *C.*

botulinum by Membré *et al.* (2009) and Membré (2009) and the QMEA for *B. cereus* in chapter 7.

It is important to note several differences between the two exposure assessments. First, *C. botulinum* has a much lower threshold of concern (i.e. growth) than *B. cereus* ($\geq 10^5$ CFU/g). Second, the tolerable number of 'risky' packages is also considerably lower for *C. botulinum* ($1 \cdot 10^{-6}$ or 1 per million) compared to that of *B. cereus* (current: 0.475% or 4750 per million). And thirdly, both assessments include temperature abuse by the consumer, but the *C. botulinum* assessment assumes that all REPFEDs are consumed at the end of shelf life, while the assessment for *B. cereus* uses a 'time-to-consumption' distribution. These three differences, means that the risk-curves presented for *C. botulinum* in this chapter are based on a 'stricter' model than those for *B. cereus*, which is only logical given the severe risk posed by *C. botulinum* compared to the mild symptoms usually caused by *B. cereus* (Carlin *et al.*, 2000a).

The risk-boundaries for *B. cereus* (green) and *C. botulinum* (blue), presented in figure 9.6 clearly show that for the same shelf life *B. cereus* requires lower pH-values and/or higher pasteurisation treatments. For example, a pasteurisation of 10 minutes at 90°C of a product with pH 6.0 allows a shelf life of more than 50 days with respect to *C. botulinum* (Figure 9.6a). For *B. cereus* the same combination of pH and pasteurisation only allows a shelf life of 25 days. The length of the heat treatment does not reduce the difference between both bacteria. A pasteurisation of 15 minutes at 87°C of a product with pH 6.0 allows a shelf life of 52 days with respect to *C. botulinum*, while for *B. cereus* the maximum shelf life under the same conditions is 25 days (Figure 9.6b). At low pH (5.7) the results converge, while at high pH (6.3) the difference is considerably larger. Although even at low pH there is still a considerable difference between the maximum shelf life.



(a) Heating time = 10 min

(b) Heating time = 15 min

Figure 9.6: Iso-risk curves for different pH-values (◊ 5.7, □ 6.0 and ○ 6.3) and two microorganisms: *B. cereus* (green) and *C. botulinum* (blue). Maximum shelf life as a function of heat treatment temperature to obtain the same % of 'risky packs' for *B. cereus* as in the baseline scenario (a_w: 0.99, heat treatment time: 10min).

9.5 Conclusions

The iso-risk curves presented in this chapter can be used for product development; to determine combinations of pH, pasteurisation time, pasteurisation temperature and shelf life that achieve the same risk as the current baseline scenario (±0.48% 'risky' packages, see chapter 7, with a_w 0.99 and variable storage temperature profile during the shelf life). The curves show that pH is the most significant parameter, i.e. the consumer exposure to *B. cereus* from REPFEDs is most 'sensitive' to pH changes. Therefore, pH is a critical factor, which needs to be monitored and controlled during production and in the finished product. However, REPFEDs are complex products and the pH in a product and in the different components will change over time until an equilibrium is reached. For example, if a product contains both a tomato sauce (pH ± 5.6) and a béchamel sauce (pH ± 6.2), what will eventually be the pH of the *B. cereus* environment and how will this pH change during shelf life? This pH evolution of a food product adds an additional measure of uncertainty to the iso-risk curves and this uncertainty is currently not included in the QMEA. The iso-risk curves also show that increasing pasteurisation temperature has more effect on the maximum shelf life than increasing pasteurisation time.

A second application is the use of risk-boundaries. The comparison between *C. botulinum* and *B. cereus* showed that the combination of pH and heat treatment required to prevent unacceptable consumer risks, are much stricter for *B. cereus* than for *C. botulinum*. This means that when a product-process combination is designed based on the consumer exposure to *B. cereus*, it will also control the consumer exposure to *C. botulinum*. However, while *C. botulinum* may be easier to control than *B. cereus*, given the high lethality associated with *C. botulinum* infections, both have to be considered when determining process/product combination for REPFEDs.

The iso-risk curves can be used to virtually 'experiment' with product-process combinations and to determine what are the options based on the consumer exposure. However, other pathogenic or spoilage microorganisms still have to be controlled. It is therefore necessary to check if the selected product-process combination can also control these microorganisms and whether this combination is feasible. This feasibility pertains to processing conditions (e.g. is 1.8 sec at 85°C realistic to achieve) and other microorganisms (e.g. will 1.8 sec at 85°C inactivate *L. monocytogenes*?). Another factor to consider is the effect on the sensorial aspects of the product, often a minimal heat treatment is required to make the food palatable. As previously noted by Membré *et al.* (2009), these results are very encouraging from a risk management point of view, but should be interpreted with due care and it is vital to experimentally validate any product-process combination before applying it at an industrial level.

Chapter 10

Conclusions

10.1 REPFEDs, complexity at its finest

Refrigerated and processed foods of extended durability (REPFEDs) are challenging products to define. Throughout this research the complexity of these products became ever more clear, as the number of possible product-process for REPFEDs currently on the market, is seemingly endless. Products range from 'simple' mashed potatoes to complex meals such as lasagne; package sizes range from 300g to 3kg; shelf lives range from 7 to 70 days; pasteurisation conditions range from 2 minutes at 70°C over 3 hours at 87°C to 10 minutes at 90°C; packaging can be done before pasteurisation, or after, or even both; the intended reheating by the consumer differs in method (microwave, classic oven, au bain marie), time and temperature. In short, there are no two REPFEDs, which are alike. The scope of this PhD was limited to REPFEDs that are industrially produced and packaged by large manufacturers or SMEs and distributed via supermarkets. REPFEDs that are distributed via small retailers (e.g. butchers) or caterers are not included. No outbreaks linked to industrially produced REPFEDs have yet been reported, although there is anecdotal evidence that a number of individual cases occur (personal communication from REPFED producers).

Fortunately, REPFEDs have some common characteristics: (i) they are designed for consumer convenience, require little effort to prepare and (ii) their microbial safety is assured using product formulation (pH, a_w), pasteurisation, packaging (MAP, vacuum or air) and cold storage. The pasteurisation inactivates a significant part of the competing microorganisms and pH and a_w are usually slightly modified, but not to the extent that they can completely inhibit bacterial growth. Finally, the reheating by the consumer does not guarantee inactivation of pathogenic microorganisms. The combination of these characteristics makes them a potential playground for the more heat resistant, less competitive pathogenic bacteria.

Three pathogens are commonly associated with REPFEDs: *L. monocytogenes*, *C. botulinum* and *B. cereus* (Mossel & Struijk, 1991; Carlin *et al.*, 2000a; Reij *et al.*, 2004). The first one differs from the other two, because it is not a spore former. Hence, *L. monocytogenes* does not have the potential to survive a correctly applied pasteurisation treatment. However, if the product is susceptible to recontamination after pasteurisation, then *L. monocytogenes* is a potential risk. Such recontamination can occur if the package is sealed after pasteurisation, if some ingredients are added after pasteurisation or if the product is repacked. Therefore, these processing steps are usually performed in a high care environment. The second pathogen, *C. botulinum*, is a spore former that produces a highly lethal toxin. Fortunately, *C. botulinum* strains are generally either heat-resistant and mesophilic or (more) heat sensitive and psychrotrophic. Applying the classic P_{90} =10 (90°C, 10min) will inactivate the psychrotrophic strains and cold storage will prevent

the surviving mesophilic strains from growing (Table 1.2, p. 15). The third pathogen, *B. cereus*, is more difficult to control. Even the psychrotrophic strains are still fairly heat resistant and can easily survive the P_{90} =10 treatment used to inactivate *C. botulinum*. Fortunately, not all *B. cereus* strains are psychrotrophic or produce the emetic toxin.

In the United States and the United Kingdom, *C. botulinum* is most frequently the organism of interest in REPFEDs, while in continental Europe, attention is divided between *C. botulinum* and *B. cereus*. As an example, the number of publications in Web of Science was compared for *B. cereus* and *C. botulinum* in Europe (10 countries) and in the USA, the UK and Australia, between 1978 and 2013. For Europe the terms "botulinum food" or "botulism food" gave 28 results, compared to 65 for "cereus food". For USA, UK and Australia there were respectively 73 and 41 results. A first reason, can be historical. The UK and USA have long history of canning, which focusses on the elimination of *C. botulinum*. By contrast, Europe has a history of pasteurisation of dairy products, in which *B. cereus* is a pathogen of concern. A second possible reason is the use of nisin, which can inhibit growth of *B. cereus* (Kim *et al.*, 2008). In the European Union, the use of nisin as a preservative is only allowed in specific egg and dairy products (Anonymous, 2008; DG Sanco, 2013). In the USA, nisin is allowed as an additive in REPFEDs, in concentrations up to 250ppm (Anonymous, 2012) and is generally regarded as safe (GRAS) (FDA, 2000). A potential strategy for REPFEDs companies might be to submit an application to the European Commission to authorise the use of nisin in REPFEDs. It is then the task of the European Food Safety Agency to assess the safety of nisin as a food additive. However, the use of nisin might conflict with the consumers' desire for 'clean label' foods (i.e. foods without preservatives). Moreover, the use of nisin cannot be the entire explanation, since nisin has been shown to affect both *Bacillus* and *Clostridium* species (Thomas *et al.*, 2001). A third cause can be the fact that *B. cereus* symptoms, in contrast to *C. botulinum*, are often mild and that *B. cereus* is therefore underreported (Scallan *et al.*, 2011). Because *B. cereus* usually causes only mild symptoms, it may give less reason to file a liability claim in comparison with the lethal toxins of *C. botulinum*. Given that food safety litigation is more common in the US than in the EU and that liability insurance in the US cost 20 times more than in Europe (Buzby & Frenzen, 1999; Loureiro, 2008), risk avoidance is an understandable behaviour.

There are several reasons for focussing research on *B. cereus*: its high heat resistance (van Asselt & Zwietering, 2006), its ability to grow at low temperature (Samapundo *et al.*, 2011b), its high prevalence in REPFEDs (Samapundo *et al.*, 2011b; Choma *et al.*, 2000b; Del Torre *et al.*, 2001) and the fact that it is probably underreported as a foodborne pathogen (Scallan *et al.*, 2011). This underreporting has several reasons. First, a *B. cereus* food infection or intoxication causes only

mild symptoms. Second, *B. cereus* is not a zoonotic organism. Therefore, monitoring only really took off fairly recent (2004-2005). This increased attention, combined with a number of severe cases (Dierick *et al.*, 2005; Naranjo *et al.*, 2011), have increased awareness about *B. cereus* as a foodborne pathogen.

The scope of this PhD, although focussed on *B. cereus*, was broad (and complex). Previous exposure assessments for *B. cereus* in REPFEDs, focussed on a single product. For example, Nauta (2001) and Afchain *et al.* (2008) developed an exposure assessment for courgette puree; Malakar *et al.* (2004) developed an exposure assessment for vegetable puree. The Quantitative Microbiological Exposure Assessment (QMEA) presented in this PhD used the same methodology as Nauta (2001) but was designed to be more broadly applicable. It was not designed for one product, but is adaptable to any REPFEDs, irrespective of the recipe or production process (given that it is in-pack pasteurised). Additionally, it also incorporates a model for *B. cereus* lag time (germination and cell lag) as a function of heat treatment, and – for certain processes – it is less reliant on expert opinion (e.g. consumer behaviour) (Nauta, 2001; Nauta *et al.*, 2003; Afchain *et al.*, 2008). Thanks to the cooperation with the Belgian REPFEDs industry, a considerable amount of new microbiological data and information on processing times, temperatures and practices was available.

10.2 Data gaps – former and current

In the course of this PhD, several data gaps were identified. Some were resolved with new experimental or survey data, while others remain unsolved.

First, there was little data available on the *B. cereus* **contamination in raw materials and during the production process**. Although some literature data is available, it was not sufficient to cover the various steps of the entire production process. To gather data, two sources were used; The companies own databases, which contained analysis used for HACCP verification, and a systematic microbiological sampling plan that was performed in five REPFED production sites at the start of this PhD. The results showed that the current microbial safety and quality of REPFEDs is good. The assessment also revealed a large diversity in raw materials, which was a challenge for modelling raw material contamination. This issue was overcome by dividing the raw materials in five groups: (i) dry herbs and spices, (ii) starch components, (iii) meat, fish and dairy products, (iv) fruit and vegetable products and (v) ambient stable products. By gathering more data, a more detailed (sub)grouping may be possible. For example, if more data were available on rice products, pasta products, flour and potatoes it may be possible to split the

'starch' group in subgroups. This will allow the QMEA to start from a more detailed recipe.

Presumably, all REPFED companies in the EU perform microbiological analysis on the raw materials they use and on the products they manufacture in the framework of verification of their food safety management system (FSMS). In an ideal situation, all these data would be available in a common anonymous database. Having more data on the contamination of raw materials would considerably reduce the uncertainty that is now included in the first module of the QMEA (chapter 3). Similarly, more data on the environmental contamination could improve the recontamination model. However, to built such a database, a uniform method of sampling and analysis is required. The development or selection of sampling methods is a task for the competent authorities and/or sector organisations at a national or supranational level (e.g. EU).

Secondly, since one of the goals was to evaluate a reduction in pasteurisation intensity, it was important to consider the effect of pasteurisation on the lag time of *B. cereus* spores at low storage temperatures. Current exposure assessments for *B. cereus* only considered the inactivating effect of pasteurisation of *B. cereus* spores and do not include the effect of heat treatment on the lag time. For example, Nauta (2001) predicted lag as a function of the storage temperature, but did not include pH, a_w and heat treatment intensity. However, various studies have shown that pasteurisation also increases lag time (Membré *et al.*, 2009; Gaillard *et al.*, 2005; Laurent *et al.*, 1999). None of the predictive models available for *B. cereus* had the possibility to predict the lag time of spores (germination lag + cell lag) at low storage temperature as a function of heat treatment (see Table 1.4, p. 28). Therefore, it was decided to develop a new **lag model for heat treated *B. cereus* spores** (Chapter 4 and 5). This model was based on a collection of newly gathered laboratory data: 434 combinations of *B. cereus* strain (FF 140 or FF355), pH (5.4-6.4), a_w (0.973-0.995), storage temperature (8-30°C), heat treatment time (1-38 min) and temperature (85-90°C) (Table 5.1, p.109). The model predicts the time to detection of *B. cereus* spores as a function of these six variables and showed that pasteurisation only has a considerable impact on the lag time (germination + cell lag) of *B. cereus* spores if the pH is suboptimal (pH < 5.8). In addition to the lack of a lag model, various other problems were encountered when choosing a model for *B. cereus* growth. Some models for *B. cereus* growth, were polynomial models and therefore not suited for extrapolation. Other models were cardinal models (Rosso *et al.*, 1995), which can be extrapolated. However, to use these model the necessary parameters or cardinal values (e.g. pH_{min}, $a_{w,min}$) have to be published. This was not the case, which rendered the equations unusable. Publishing all model parameters, or better yet the complete experimental dataset, will significantly promote future advancements in predictive microbiology and in quantitative risk assessment.

A third data gap, **consumer behaviour**, only became clear during the development of the QMEA. While data about the production process was readily available by collaboration with the REPFED industry, the consumer behaviour with respect to REPFEDs was largely unknown. During the development of the QMEA, a preliminary sensitivity analysis was performed and the parameter with the largest effect on the output was the time a product spent in a consumer refrigerator. At the time, this parameter was purely based on expert opinion (Nauta, 2001) and was very uncertain. Therefore, it was decided to gather new data on this aspect of consumer behaviour. Using a consumer survey, it became clear that most REPFEDs are stored for only a fairly short time in the consumer refrigerator (0-7 days), despite the fact that the shelf lives are much longer (10 - 45 days).

Inevitably, **certain data gaps still remain**. Table 10.1 gives an overview of the different sources of uncertainty and their relative impact on the outcome of the exposure assessment. First and foremost, many aspects concerning the **behaviour and prevalence of different *B. cereus* strains** are still insufficiently known or quantifiable. For example, *B. cereus* is known to produce super-dormant spores, which will only germinate under temperature abuse (Ghosh *et al.*, 2009). However, much is still unknown about the percentage of super-dormant spores and the conditions under which they germinate. It is also known that the heat resistance of *B. cereus* spores depends on a large number of variables (pre, during and post heating) such as strain, sporulation temperature, nutrients in heating and recovery medium etc. This variability and uncertainty in the heat resistance is one of the main causes for variability in the output of the exposure assessment. Recent research has also shown that *B. cereus* had difficulty growing anaerobically at low temperature (de Sarrau *et al.* (2012, 2013) and own unpublished results). A large number of REPFEDs is packaged under modified atmosphere (MAP) and the current QMEA does not include the effect of MAP on *B. cereus*. When this data becomes available, it should be included, since this will considerably decrease the consumer exposure. In addition, there is a need for more detailed data about the physiological state (spores or vegetative cells) of *B. cereus* in the different environments and products. In the current QMEA, this physiological state is not taken into account at this stage of the model and it is assumed that all *B. cereus* present in raw materials or in the production environment are spores. However, if not all these *B. cereus* are spores, this will reduce the exposure, because vegetative cells will be inactivated during thermal preparation or pasteurisation. Another variable with respect to *B. cereus* that is not yet well defined, is the number of strains that are able to produce the emetic toxin and under which conditions this toxin is produced. In the model it is assumed that all *B. cereus* strains have the ability to produce the emetic toxin, in reality, the percentage of strains able to produce cereulide will be much lower (1-5%) (Altayar & Sutherland, 2006; Ceuppens *et al.*, 2011). Finally, the development of a com-

bined model for the lag time and growth rate of heat treated *B. cereus* spores under cold storage, would be a significant step forward in the exposure assessment of *B. cereus*.

A second, different type of data gap is the effect of storage time and temperature on the **spoilage** of REPFEDs. If a product is stored for longer and/or at higher temperatures, it is more likely to spoil. However, because spoilage is caused by many different microorganisms, this effect is difficult to quantify. In addition, it is also not yet possible to predict if a consumer will notice product spoilage and discard the product. Therefore, the current QMEA does not include spoilage or interactions between *B. cereus* and spoilage microorganisms (e.g. outgrowth of lactic acid bacteria).

The third data gap is the **consumer behaviour**. Although some data is available in literature and other aspects were treated in this PhD, there are still some important questions that need to be answered. One example is the **reheating of the products by the consumers**. Although the reheating guidelines on the label can be tested, it is difficult to assess how well consumers will follow these guidelines and what the effect is of variability in domestic appliances (e.g. microwave ovens). An even more challenging aspect is the keeping and re(re)heating of leftovers.

A fourth data gap is related to **processing conditions and product formulation**. Although plenty of data is available, some aspects are difficult to quantify. For example the effect of the time required to heat up and to cool down the product during pasteurisation on inactivation and lag is not yet quantified. Additionally, there was no data about the distribution of pH and a_w throughout a heterogeneous product. This last aspect is especially difficult for products that contain components with different pHs (e.g. tomato sauce and béchamel sauce).

The fifth and final data gap is situated on the overlap between *B. cereus* and human health: the **dose-response relation** for *B. cereus*. Although significant advances have been made in recent years, it is still difficult to determine which concentration of *B. cereus* will cause emetic or diarrhoeal symptoms. However, such a dose-response relation for *B. cereus* is an immensely complex task, given the diversity in *B. cereus* strains and the two *B. cereus* syndromes. Future research on these aspects should include the effect of food matrices on toxin production by emetic strains and the effect of the intestinal environments on survival and expression of toxin genes and the stability of toxins produced by diarrhoeal strains in the gastrointestinal tract (Ceuppens *et al.*, 2011). Most likely, a dose-response relation for *B. cereus* will not become available any time soon for a number of reasons: variability in *B. cereus* strains, complex bacteria-host cell interactions, complex regulation of toxin gene expression, lack of an easy-to-use method for enterotoxin quantification, etc. (Ceuppens, 2012).

Table 10.1: Sources of uncertainty and data gaps related to the exposure assessment for *B. cereus* in REPFEDs. Based on expert opinion and discussion in the guidance committee.

Source of uncertainty	Impact on QMEA output[a]
***Bacillus cereus* behaviour and strain diversity**	
- Prevalence of super-dormant spores	++/– –
- Ratio spores:cells present in raw materials and in the environment (compared to 100% spores in the current QMEA)	– – –
- Prevalence of psychrotrophic strains in the production environment	++/– –
- Correlation between heat resistance and psychrotrophic abilities	– –
- Prevalence of (psychrotrophic) emetic strains	– – –
- Growth under MAP and cold conditions	– –
Consumer behaviour	
- Reheating of REPFEDs by the consumer	–
- Storage and reheating of leftovers REPFEDs	+
- Survey respondents incorrectly reporting frequency of purchase or the storage time of REPFEDs (chapter 6)	+ / –
- Time-temperature profile during product shelf life	+ / –
Dose respons relation for *B. cereus*	
- For the emetic and diarrhoeal syndromes	– – –
Models / mechanisms	
- Error on lag and growth models	++ / – –
- Mechanism and rate of *B. cereus* transfer from the production environment to the product	++ / – –
- Division of lag time in germination-lag (or spore lag) and cell lag (compared to 100% spore lag in the current QMEA)	– – –
- Effect of product composition on heat resistance and lag time	+ / –
- Effect of microwave reheating on *B. cereus* spores	+ / –
- Spatial distribution of *B. cereus* in batches of intermediate products	+ / –
- Product spoilage during product shelf life	– –
Processing and product formulation	
- Non iso-thermal heating / pasteurisation	++/– –
- Modified atmosphere packaging	– – –
- pH and a_w distribution in a complex product	++ / – –

[a]: (+) means likely to cause under-estimation in the current QMEA, (-) means likely to cause over-estimation in the current QMEA. (+/-) means direction of estimation-error unsure. The number of signs is a indicator of the magnitude.

10.3 Conclusions for the different stakeholders

10.3.1 Current exposure from *B. cereus* in REPFEDs

One of the primary goals of the QMEA was to assess the current consumer exposure as a point of reference for possible risk mitigation strategies. The current exposure was estimated at 4750 (± 67) 'risky' packages in 1,000,000 packages. A 'risky' package was defined as (i) a package that originated from a batch that had contained more than 10^5 *B. cereus* cells/g during some stage of production and can contain the emetic toxin or (ii) a package that contained at least 10^5 cells or spores/g during shelf life. Of these 4750 'risky' packages, 4313 (±49) packages contained more than 10^5 *B. cereus*/g (cells or spores). The other 437 (±27) packages potentially contained the emetic toxin. An exposure of this level (4.8 in 1,000) may appear high, but the mitigating effect of several model assumptions must be taken into consideration. The most important being the effect of modified atmosphere packaging, spoilage, the actual percentage of emetic strains and the ratio of cells to spores in raw materials and the production environment. Because of these worst-case assumptions, the QMEA should not be used do determine exact exposure estimates, but rather to evaluate and compare risk mitigation strategies based on the relative difference in exposure (see chapter 8).

10.3.2 For the competent authorities

The key lessons for the authorities, responsible for food regulations, audits and inspections, are derived from chapter 7 and 8. The primary message is that end-product testing is not a good indicator of the potential exposure (Figure 7.5, p. 200), as was previously reported by Nauta (2001). The effect of consumer (mis)behaviour is simply too large, to conclude anything about the food safety of the product, based on the *B. cereus* concentration after production. However, while low concentrations at the end of production do not necessarily guarantee microbial safety, high *B. cereus* concentrations should still be avoided. If *B. cereus* concentrations at the beginning of shelf life are already high(er), less growth (and time for growth) is needed before a 'risky' *B. cereus* concentration is reached (10^5 CFU/g). Therefore, end-product testing can be considered an evaluation of the production process, but not a method to verify microbial food safety. The current microbiological guidance value for *B. cereus* in REPFEDs at the end of the production process is 10^2 CFU/g (tolerance to 10^3 CFU/g) (Uyttendaele *et al.*, 2010). According to the current QMEA, at the end of the production process, 0.75% of products exceed 10^2 CFU/g and 0.22% exceeds 10^3 CFU/g. Because of the large effect of consumer behaviour, there is no point in making these criteria more strict. Even if only one spore is present in the product

and if this product is stored under temperature abuse or for too long, there is a potential for *B. cereus* growth to hazardous concentrations. Additionally, lower guide values would require new microbiological methods since the detection limit of the classical method is 10^2 CFU/g (10 CFU/g can be obtained in some cases, when using a reduced detection limit, plating 1 ml over 3 agar plates). An alternative approach might be to take product samples on the day of production, to store them according to a standard time-temperature-profile and to test the products at the end of their shelf life, to establish whether the safety level of 10^5 CFU/g is exceeded under reasonable foreseen conditions of storage by the consumer. A possible standard method could be the EU technical guidance document (when applied to the Belgian situation) on shelf life studies for *L. monocytogenes* in RTE-foods (EU CRL for *Listeria monocytogenes*, 2008). According to this guidance document, the product should first be stored at 4°C for part (e.g. $^1/_3$) of the shelf life and then at 8°C for the remaining shelf life (e.g. $^2/_3$) (see section 2.2.2.2 on p. 47 for more information). In addition to shelf life testing and end-product testing, sampling of the production environment can be a good method to monitor the potential for *B. cereus* contamination. Since contamination during packaging and assembly was shown to be a 'critical' point during the scenario analysis in chapter 8.

When performing inspections or audits, food safety authorities have to be aware that the production of REPFEDs requires a comprehensive food safety management approach. Simply applying a pasteurisation with P_{90} =10 is no guarantee for food safety. To assure the microbial food safety of REPFEDs, good quality raw materials, environmental hygiene and adequate pasteurisation are needed. Given the erratic (or sporadic) nature of *B. cereus* contamination on raw materials and in the environment, it is unlikely that raw materials or surfaces with elevated *B. cereus* levels will be detected by sampling. Although sampling is still a good method for raising/keeping 'awareness' with personnel and to detect (major) defects in GMP and HACCP. It is also good to built a track record of negative results, in order to demonstrate the good performance of the FSMS in case of calamities or crisis situations. The **safe harbours** that are currently used in the REPFEDs production, and in other branches of food production, are based on 'older' research (1980s-1990s) and contain a considerable amount of safety margin. They are also based on a number of worst-case assumptions such as the most heat-resistant strains and high concentrations of the target bacteria. In addition, safe harbours focus all the safety control on one step (i.e. the pasteurisation process), while nowadays a holistic approach to food safety management is used and the General Food Law (Anonymous, 2002) demands the use of a complete FSMS (GMP, PRPs, HACCP, etc.) as the approach for safeguarding our food and food supply chain.

10.3.3 For the REPFED industry

The key lessons for competent authorities are also applicable to the REPFED industry. However, their goal is not only limited to food safety but also includes food quality. This means that pasteurisation is a double-edged sword: if it is too little, then it does not assure food safety and if it is too much, then it is detrimental to product quality. To assist the REPFED producers in handling this dilemma, a set of iso-risk curves is presented in chapter 9. These curves show that there are **four key variables to consider: pH, shelf life, pasteurisation time and temperature.**

As a rule of thumb, pasteurisation at higher temperatures for shorter times is more effective to prolong the lag time of *B. cereus* (chapter 5). This effect is also visible in the iso-risk curves, with warmer treatments allowing considerably shorter heat treatments. The iso-risk curves allow the producers to determine a realistic combination of pH, pasteurisation and shelf life. However, some conditions should be fulfilled: the iso-risk curves should not be extrapolated beyond the boundaries of the lag model (i.e. heat treatment between 1 and 38 minutes at 85 to 95°C), and the shelf life should always be validated in the product. When reducing the heat treatment, it is important to take other pathogenic microorganisms into account. For example, Figure 9.2 (p.234) demonstrates that at pH 5.6, REPFEDs require virtually no heat treatment to obtain the same exposure as the baseline scenario (1.8 sec at 85°C). However, it is unlikely that this heat treatment will inactivate other pathogenic microorganisms such as *L. monocytogenes*. The practical feasibility of applying such a short heat treatment to a solid product is also questionable.

The iso-risk curves also show that shorter shelf lives allow the use of less intense heat treatments. Given that ±90% of REPFEDs are consumed within one week after purchase, the added value of long shelf lives (>21 days) can be questioned, both from a food safety and a marketing point of view. However, shorter shelf lives have one downside. According to the consumer survey, shorter shelf lives mean that more products will be discarded instead of consumed, possibly because the products are forgotten in the refrigerator until after the 'use by' date (Table 6.6, p.151). This is not necessarily a direct disadvantage for the producer, but it is a loss for the consumer, a source of food waste and therefore not a sustainable practice. Consumer education may also improve this aspect of consumer behaviour.

Additionally, REPFED producers should be aware that both raw material quality and good hygiene can help to increase product quality and safety. Unfortunately, further improvement of good hygiene caused a smaller reduction in exposure (scenarios 2 and 7, Table 6.6, p.151) compared to the large increase in exposure caused by non compliance to good hygiene practices (scenarios 3 and 8). If hygiene at the packaging and assembly stage is not controlled, this can

lead to a five-fold increase in exposure (4750→ 24291 'risky' packages per 10^6 packages), while perfect hygiene will only halve the exposure (4750→2035). Presumably, low hygiene will also cause several other (more noticeable) quality defects. The current level of hygiene is acceptable, but needs to be monitored and kept on track.

REPFED producers can use the models and the exposure assessment in this PhD to assess if a product/process combination is a good choice, i.e. that this combination does not give very short lag times and/or a higher exposure. This approach will permit a more efficient investment of resources in product development and process optimisation. However, all the product-process combinations will still have to be validated in the actual product and validation has to be done by challenge testing according to a standard protocol. Any such protocol has to fulfil a number of requirements. First, it is recommended that the protocol is performed with a cocktail of worst-case *B. cereus* strains (i.e. heat-resistant, cold growing *B. cereus* strains). Second, the product has to be inoculated with spores (and not vegetative cells) and subsequently submitted to a heat treatment. This in-product heat treatment assures that the effect of the heat treatment is as close to reality as possible. Finally, the product has to be stored for the duration of its shelf life at a standard time-temperature-profile. Preferably, this profile should be identical to that for challenge testing of *L. monocytogenes* (EU CRL for *Listeria monocytogenes*, 2008). Evidently, such a standard protocol should be agreed upon by the industry and the competent authorities and should include some reasonably foreseeable abuse without including extreme and irresponsible consumer behaviour.

10.3.4 For all stakeholders

All stakeholders in the production of safe REPFEDs (i.e. producers, competent authorities, retailers and consumers) have to be aware that **consumer behaviour is the key cause of exposure according** to the current sensitivity and scenario analysis (chapter 7 and 8). Consumer with a refrigerator operating at temperatures higher than 8°C, are 13 times more likely of consuming REPFEDs with a 'risky' *B. cereus* concentration. If all consumer refrigerators would be at the correct temperature (≤8°C), the exposure to *B. cereus* from REPFEDs is estimated to be ±80% lower (4750→929 'risky' packages per 10^6 packages) (scenario 15, chapter 8). It is reasonable to assume that this also applies to other microorganisms that thrive in case of temperature abuse. It may be worthwhile, to invest in consumer education about food safety. However, the question is whether consumers will listen or not. A possibility might be to target the 'next' generation of consumers and give a number of food safety classes during primary and secondary school. An alternative, more direct approach, could be to demand that all consumer refrigerators have

a built-in temperature display. An even more direct solution, would be to require that refrigerators cannot operate at temperatures higher than 8°C. However, such a requirement would be difficult to achieve and impossible to verify (e.g. what with older refrigerators?). Both consumer education on safe food handling practices and regulating refrigerator displays or operating temperatures will require considerable amounts of time and money and has to be seen as a long term objective.

10.3.5 For risk assessors

During this PhD, the importance of consumer behaviour became clear. Both in terms of use and in terms of abuse (time to consumption, frequency of purchase,...). The EU regulation on microbiological criteria for foodstuffs states that the food safety criteria should be met under **reasonably foreseeable conditions of distribution, storage and use** (Anonymous, 2005). This raises the question "what is reasonably foreseeable abuse?" and should a risk assessment or shelf life test include 'unreasonably abusive' consumers. When performing a risk or exposure assessment, these consumers have to be included. But their effect on the risk has to be clearly identified and quantified. However, when performing a challenge test or shelf life experiment, including these consumers will lead to unrealistically short shelf lives or over-pasteurised food products. Is is therefore important that **reasonable foreseeable abuse is accurately defined and fixed**, because the current situation allows much uncertainty in the interpretation. As an example, for the temperature in the consumer refrigerator, the 75% of consumer refrigerator temperatures (8°C) could be used as the limit of reasonableness (Vermeulen *et al.*, 2011).

In the same line of reasoning, the argument can be made that **the difference between a 'use by' date and a 'best before' date is a matter of perspective**. If a consumer respects the storage guidelines on the label (e.g. "store at 4°C or below") and if these guidelines are sufficiently strict to prevent growth of psychrotrophic *B. cereus* and *C. botulinum* strains (i.e. not "store at 7°C or below"), then the date on an in-pack pasteurised REPFEDs can be considered a 'best before' date. This is the case, because recontamination with *L. monocytogenes* is not possible after in-pack pasteurisation and *C. botulinum* and *B. cereus* are highly unlikely to grow or produce toxins at 4°C. However, it is unlikely that all consumer refrigerators will be able to achieve these low temperatures. Therefore, consumer abuse – reasonable foreseeable or other – must be taken into account, and the date has to be considered a 'use by' date. This corresponds to the Codex Committee on Food Hygiene (CCFH) (1999) definition of REPFEDs, which states that REPFEDs "are susceptible to out- growth of pathogenic microorganisms" (p.2).

At first sight, the conceptual ICMSF equation (Equation 7.2, p.158) and the **setting of PO/FSO** is

a straightforward task. However, in practice it is much more complex, because of the complexity of the production process and the large variability caused by consumer behaviour (Figure 10.1). As a proof-of-concept, an attempt was made to determine two Performance Objectives (POs) to reach a specified Food Safety Objective (FSO). The FSO was specified as: "maximum one package with more than 10^5CFU/g per 10,000 packages at the moment of consumption" or in other words, max 0.01% 'risky' packages. This FSO is 50 times lower than the current exposure according to the QMEA (0.48%). As indicated on Figure 10.1 the FSO is applicable at the moment of consumption. The first PO (PO_1) was a maximum *B. cereus* concentration (CFU/g) in raw materials, the second (PO_2) was a maximum *B. cereus* concentration (CFU/package) on packaged products before pasteurisation. Several values were tested for both POs. For the PO_1, no value could be obtained that guaranteed achieving the FSO. Even if PO_1 was set to absence of *B. cereus* on raw materials, i.e. when the raw materials did not contain any *B. cereus* spores, the FSO could not be reached. Even with this unrealistic PO, the percentage of 'risky' REPFEDs is still 0.34% (34 packages per 10,000 packages). The cause for this limited effect of raw material quality was the recontamination during handling of raw materials and during packaging and assembly. Hence, reducing raw material contamination has to be combined with further improving good hygiene practices. PO_2 was equally problematic to determine. Even if the *B. cereus* concentration before pasteurisation was set to 1 *B. cereus* per package in only half the packages (the other not containing *B. cereus*), the exposure was still 6 packages per 10^4 packages (0.06%). The FSO could only be reached when the *B. cereus* prevalence and concentration before pasteurisation were unrealistically low. For example, a PO_2 with prevalence 10% and a concentration of 1 CFU/pack in contaminated packages was one of the combinations of prevalence and concentration adequate to achieve the FSO. In addition to being unrealistically low, this PO is also impossible to verify and enforce. To reach the set FSO, very strict POs were needed because of the impact of recontamination during processing and consumer behaviour during the product shelf life. A package that contains only one *B. cereus* spore at the end of production, which is subsequently stored under temperature abuse can still contain too high *B. cereus* concentrations at the time of consumption. As a final remark, determining a value for a PO required a large amount of trial and error. As explained by Havelaar *et al.* (2004), it is impossible to deconstruct the probability distributions and therefore it is impossible to solve the ICMSF equation for a probabilistic model (ICMSF, 2002). Despite these issues, the ICMSF equation is a good concept to illustrate the basic logic of food chain safety.

The development of the QMEA demonstrated that to perform a realistic and data-based exposure assessment, a multi-disciplinary team is needed. This team should contain food microbiologists, risk assessors, predictive modellers and **industry representatives**. Without industry coopera-

tion and industry data it would have been **impossible** to perform a realistic quantitative risk assessment. The food industry has a wealth of data on the microbiological contamination and the processing conditions of their products. The current assessment was made possible by the cooperation of several REPFEDs companies. Without their cooperation, insufficient data would haven been available. For future risk assessments, the incorporation of industry partners on a national or European level is recommended. However, as with all science-industry cooperation: risk-assessor may accept data or input from the industry, but independency in generating and interpreting the results should be assured by transparent communication and peer-review.

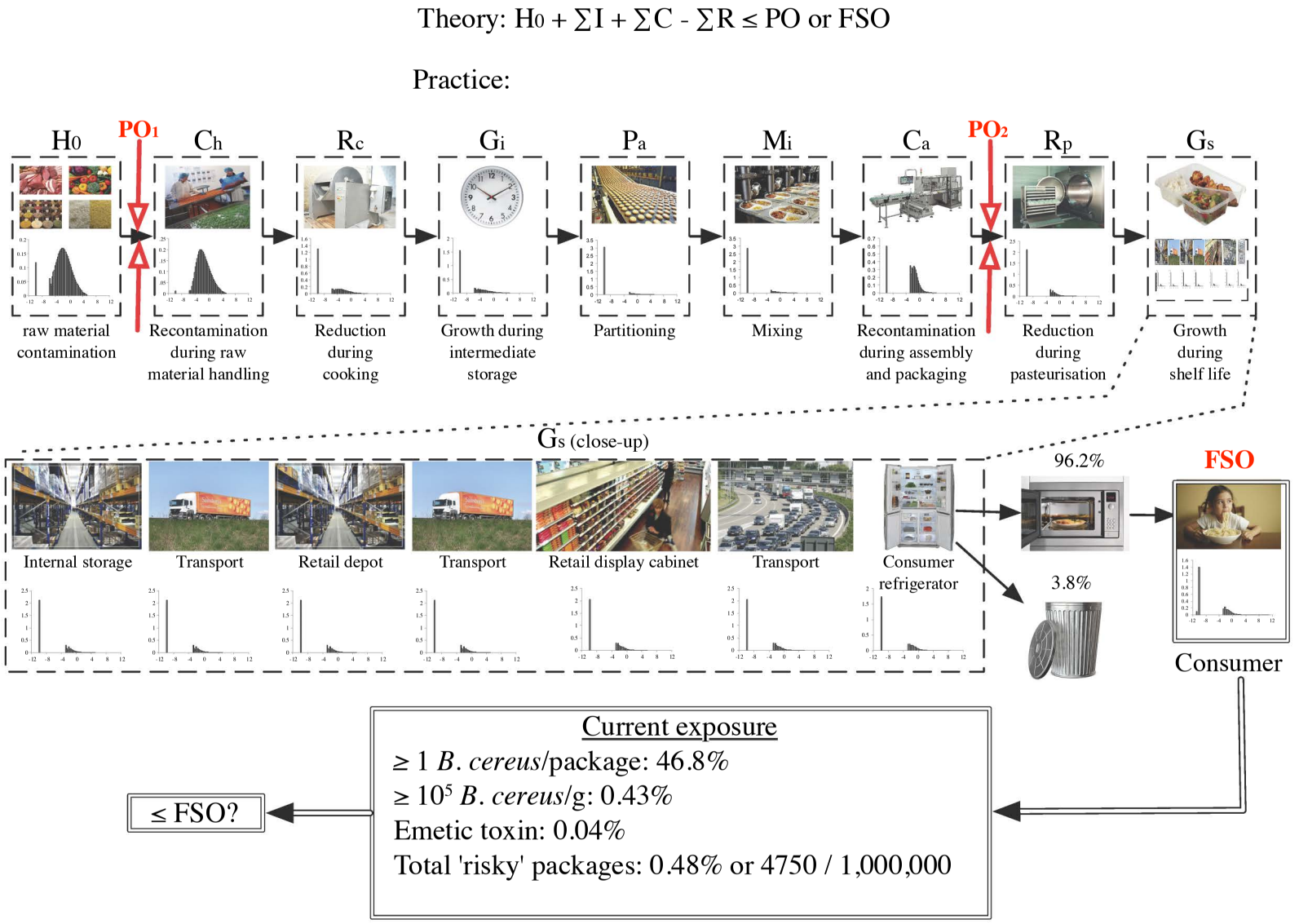


Figure 10.1: Illustration of the ICMSF equation (Equation 7.2, p.158) and the PO/FSO approach applied to the REPFEDs production and distribution process. (Distributions represent the *B. cereus* at the different steps according to the baseline scenario (Chapter 7).

10.4 Perspectives for future research

Based on the findings of this PhD and the data gaps that were encountered, a number of avenues for future research could be identified. A primary topic is the development of a dose-response relation for *B. cereus* spores, *B. cereus* cells and the different toxins (Ceuppens *et al.*, 2012). A dose-response relation would allow the translation of an exposure assessment into an actual risk assessment. Another important topic of study is bacterial spores, both on a cellular and a macroscopical level. On a cellular level, more information about the mechanisms of sporulation, germination and heat inactivation is needed. Preferably this knowledge should then be incorporated in predictive model. However, the question is, whether a structure that is inherently variable in order to survive, can be adequately modelled. On a macroscopic level, more information is needed about the effect of heating up and cooling down on inactivation of spores. Although the current D-/z- approach is widely used, it is an over-simplification of reality. A related topic is the effect of microwaves and microwave heating on bacterial spores, both at a consumer and a production level. Especially since microwave heating has been reported to cause more inactivation and more damage to bacterial spores than boiling (Kim *et al.*, 2009).

A different area of research is the use of second order Monte Carlo simulations, to determine the levels of uncertainty and variability in the exposure assessment. However, while this may be of scientific interest, the utility to the food industry is debatable. The current exposure assessment, although only first order (i.e. no differentiation between variability and uncertainty) is already very complex. Understanding the development, the assumptions and the implications of these assumptions on the exposure of a second order model, will be even more difficult for a food business operator. Especially, since they usually are not risk assessment experts and a tool that is not well understood, is likely to be less (well) used. It is important to consider this double effect of model complexity. On one hand it makes the model predictions more accurate (although not necessarily) (Zwietering, 2009), but on the other hand it makes the model less accessible for non-expert users. In extreme cases, it may even only be clear to the model developer(s). To prevent this from happening it is vital to provide a clearly written, comprehensive account of the model and its assumptions.

A final avenue of research is the measurement and steering of consumer behaviour. As shown in chapters 7 and 8, consumer behaviour is a key component in food safety. Changing the consumer food safety culture will require consumer education ('why') and consumer training ('how'). Consumers have to be made aware why their behaviour causes risk and what they should (or should not) do in order to avoid such risk. Both these processes should be risk-based, i.e. they should target the most influential behaviour first (Yiannas, 2009). In the case of REPFEDs, the primary

target should be the consumer refrigerator temperature. To accurately change the consumer behaviour, it is vital that any communication about the food safety risks is based on the consumers perceptions and concerns (Cope *et al.*, 2010; Frewer, 2004). Since consumers tend to underestimate the effect of their personal behaviour on the risk and overestimate the risk of technological advancements (Verbeke *et al.*, 2007), this will not be a simple task. The communication to the consumer should also include a description of the risk uncertainty. While experts commonly believe that consumers cannot grasp the implications of risk uncertainty, consumers have the impression that this information is being kept from them. Hence, if a risk is communicated without uncertainty and the media provides evidence to the contrary, consumers will loose trust in risk assessors and communicators. Finally, since risk communication (and hence consumer education) has to be based on the consumers perceptions and information needs, the communication should be tailored to specific regional/cultural groups. This implies that consumer education may be more effective at national or even, regional level (Cope *et al.*, 2010).

Reference list

Reference list

Abee, T., Groot, M. N., Tempelaars, M., Zwietering, M., Moezelaar, R. & van der Voort, M. (2011). Germination and outgrowth of spores of *Bacillus cereus* group members: Diversity and role of germinant receptors. *Food Microbiology*, 28(2):199–208.

Adams, M. (2005). Detecting susublethal ddamage cells. In Griffiths, M., editor, *Understanding pathogen behaviour*. Woodhead Publishin Ltd, Cambridge, England.

Advisory Commitee on the Microbiological Safety of Food (ACMSF) (1992). Report on vacuum packaging and associated processes.

Afchain, A. L., Carlin, F., Nguyen-the, C. & Albert, I. (2008). Improving quantitative exposure assessment by considering genetic diversity of *B. cereus* in cooked, pasteurised and chilled foods. *International Journal of Food Microbiology*, 128(1):165–173.

Afchain, A. L., Derens, E., Guilpart, J. & Cornu, M. (2005). Statistical modelling of cold-smoked salmon temperature profiles for risk assessment of *Listeria monocytogenes*. *Acta Horticulturae*, 674:383–388.

AFF (2000). Recommandations de l'AFF concernant la conservation des denrées réfrigérées au niveau des ménages. Technical report, Association Française du Froid.

AFNOR (2010). Nf v01-003 - Hygiène des aliments - lignes directrices pour la réalisation de tests de vieillissement microbiologique - aliments périssables et très périssables réfrigérés.

Agata, N., Ohta, M., Mori, M. & Isobe, M. (1995). A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. *Fems Microbiology Letters*, 129(1):17–19.

Agresti, A. (2002). *Categorical Data Analysis*. Wiley, New York.

Albarracin, W., Sanchez, I., Grau, R. & Barat, J. (2011). Salt in food processing; usage and reduction: a review. *International Journal of Food Science and Technology*, 46:1329–1336.

Altayar, M. & Sutherland, A. D. (2006). *Bacillus cereus* is common in the environment but emetic toxin producing isolates are rare. *Journal of Applied Microbiology*, 100(1):7–14.

Amezquita, A., Weller, C. L., Wang, L., Thippareddi, H. & Burson, D. E. (2005). Development of an integrated model for heat transfer and dynamic growth of *Clostridium perfringens* during the cooling of cooked, boneless ham. *International Journal of Food Microbiology*, 101(2):123–144.

Anderson, N., Larkin, J. W., Cole, M. B., Skinner, G. E., Whiting, R. C., Gorris, L. G. M., Rodriguez, A., Buchanan, R., Stewart, C. M., Hanlin, J., Keener, L. & Hall, P. (2011). The food safety objective approach for controlling *Clostridium botulinum* growth and toxin production in commercially sterile foods. *Journal of Food Protection*, 74(11):1956–1989.

Ankolekar, C. & Labbé (2009). Survival during cooking and growth from spores of diarrheal and emetic types of *Bacillus cereus* in rice. *Journal of Food Protection*, 72(11):2386–2389.

Anonymous (2002). Regulation (EC) No 178/2002 of the european parliament and of the council of 28th january 2002 laying down the general principles and requirements of food law, establishing the european food safety authority and laying down procedures in matters of food safety.

Anonymous (2005). Commission regulation (EC) No 2073/2005 of 15 november 2005 on microbiological criteria of foodstuffs. *Official Journal of the European union*, 338:1–26.

Anonymous (2008). regulation (EC)) No 1333/2008 of the European parliament and of the council of 16 december 2008 on food additives. *Official Journal of the European union*, 354:16.

Anonymous (2009). Koninklijk besluit van 26 april 2009 betreffende microbiologische criteria voor voedingsmiddelen.

Anonymous (2012). code of federal regulations - title 21 - food and drugs, part 184 – direct food substances affirmed as generally recognized as safe subpart b – listing of specific substances affirmed as GRAS, sec. 184.1538 nisin preparation. URL `http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=184.1538&SearchTerm=nisin`. Consulted June 1, 2013.

Anonymous (2013). Rapid Alert System for Food and Feed (RASFF). URL `http://ec.europa.eu/food/food/rapidalert/rasff_portal_database_en.htm`. Consulted May 31, 2013.

Appert, N. (1810). Le livre de tous les ménages, ou l'art de conserver, pendant plusieurs années, toutes les substances animales et végétales (translated by K. G. Bitting, chicago, 1920). In Goldblith, S., Joslyn, M. & Nickerson, J., editors, *Introduction to thermal processing of foods. (1961)*. AVI, Westport, CT.

Arnesen, L. P. S., Fagerlund, A. & Granum, P. E. (2008). From soil to gut: *Bacillus cereus* and its food poisoning toxins. *Fems Microbiology Reviews*, 32(4):579–606.

Arnoldi, A. (2002). Thermal processing and nutritional quality. In Henry, C. & Chapman, C., editors, *The Nutrition Handbook for Food Processing*, pp. 265–286. Woodhead, Cambridge, England.

Augustin, J. C. (2011). Challenges in risk assessment and predictive microbiology of foodborne spore-forming bacteria. *Food Microbiology*, 28:209–213.

Augustin, J. C. & Carlier, V. (2000). Mathematical modelling of the growth rate and lag time for *Listeria monocytogenes*. *International Journal of Food Microbiology*, 56(1):29–51.

Augustin, J. C., Zuliani, V., Cornu, M. & Guillier, L. (2005). Growth rate and growth probability of *Listeria monocytogenes* in dairy, meat and seafood products in suboptimal conditions. *Journal of Applied Microbiology*, 99(5):1019–1042.

Aureli, P., Di Cunto, M., Maffei, A., De Chiara, G., Franciosa, G., Accorinti, L., Gambardella, A. & Greco, D. (2000). An outbreak in Italy of botulism associated with a dessert made with mascarpone cream cheese. *European Journal of Epidemiology*, 16(10):913–918.

Bae, Y., Kim, B. R., Lee, S., Cha, M., Park, K., Chung, M. & Ryu, K. (2011). Growth and predictive model of *Bacillus cereus* on blanched spinach with or without seasoning at various temperatures. *Food Science and Biotechnology*, 21(2):503–508.

Bahk, G. J., Todd, E. C. D., Hong, C., Oh, D. & Ha, S. D. (2007). Exposure assessment for *Bacillus cereus* in ready-to-eat kimbab selling at stores. *Food Contro*, 18(6):682–688.

Baker, J. M. & Griffiths, M. W. (1993). Predictive modeling of psychrotrophic *Bacillus cereus*. *Journal of Food Protection*, 56(8):684–688.

Ball, C. O. (1943). Short time pasteurization of milk. *Industrial & Engineering Chemistry*, 35 (1):71–84.

Baranyi, J. & Pin, C. (2004). Modeling the history effect on microbial growth and survival: deterministic and stochastic approaches. In McKellar, R. & Lu, X., editors, *Modeling microbial responses in food*, pp. 285–301. CRC Press, London.

Baranyi, J. & Roberts, T. A. (1994). A dynamic approach to predicting bacterial-growth in food. *International Journal of Food Microbiology*, 23(3-4):277–294.

Baranyi, J. & Tamplin, M. L. (2004). Combase: A common database on microbial responses to food environments. *Journal of Food Protection*, 67(9):1967–1971.

Baril, E., Coroller, L., Couvert, O., Leguerinel, I., Postollec, F., Boulais, C., Carlin, F. & Mafart, P. (2012). Modeling heat resistance of *Bacillus weihenstephanensis* and *Bacillus licheniformis* spores as function of sporulation temperature and pH. *Food Microbiology*, 30(1):29–36.

Barker, G. C., Malakar, P. K. & Peck, M. W. (2005). Germination and growth from spores: variability and uncertainty in the assessment of food borne hazards. *International Journal of Food Microbiology*, 100(1-3):67–76.

Bean, D., Bourdichon, F., Bresnahan, D., Davies, A., Geeraerd, A., Jackson, T., Membré, J. M., Pourkomailian, B., Richardson, P., Stringer, M., Uyttendaele, M. & Zwietering, M. (2012). Risk assessment approaches to setting thermal processes in food manufacture. Technical report, ILSI Europe.

Beattie, S. H. & Williams, A. G. (2002). Growth and diarrhoeagenic enterotoxin formation by strains of *Bacillus cereus* in vitro in controlled fermentations and in situ in food products and a model food system. *Food Microbiology*, 19(4):329–340.

Bell, C. & Kyriakides, A. (2002). *Listeria monocytogenes*. In Blackburn, C. D. W. & McClure, P., editors, *Foodborne pathogenes*. Woodhead publishing Ltd.

Benedict, R. C., Partridge, T., Wells, D. & Buchanan, R. L. (1993). *Bacillus cereus* - aerobic growth-kinetics. *Journal of Food Protection*, 56(3):211–214.

Bennik, M. H. J., Smid, E. J., Rombouts, F. M. & Gorris, L. G. M. (1995). Growth of psychrotrophic foodborne pathogens in a solid surface model system under the influence of carbon dioxide and oxygen. *Food Microbiology*, 12(6):509–519.

Bigelow, W. D. (1921). Logarithmic nature of thermal-death-time curves. *Journal of Infectious Disease*, 29:528–536.

Bottone, E. (2012). Reply to "can *Bacillus cereus* food poisoning cause sudden death?". *Journal of Clinical Microbiology*, 50(11):3817.

Bowers, D. (2000). Cooking trends echo changing roles of women. *Food Review*, 23:23–29.

Braconnier, A., Broussolle, V., Perelle, S., Fach, P., Nguyen-The, C. & Carlin, F. (2001). Screening for *Clostridium botulinum* type A, B, and E in cooked chilled foods containing vegetables and raw material using polymerase chain reaction and molecular probes. *Journal of Food Protection*, 64(2):201–207.

Brennan, M., McCarthy, M. & Ritson, C. (2007). Why do consumers deviate from best microbiological food safety advice? an examination of high-risk' consumers on the island of ireland. *Appetite*, 49(2):405–418.

Business Insights (2006). Growth strategies in ready meals: consumer drivers, new meal opportunities, innovation and format and flavor trends.

Busschaert, P., Geeraerd, A. H., Uyttendaele, M. & Van Impe, J. F. (2011). Hierarchical bayesian analysis of censored microbiological contamination data for use in risk assessment and mitigation. *Food Microbiology*, 28(4):712–719.

Buzby, J. & Frenzen, P. (1999). Food safety and product liability. *Food Policy*, 24(6):637–651.

Byrne, B., Dunne, G. & Bolton, D. J. (2006). Thermal inactivation of *Bacillus cereus* and *Clostridium perfringens* vegetative cells and spores in pork luncheon roll. *Food Microbiology*, 23(8):803–808.

Caipo, M. L., Duffy, S., Zhao, L. & Schaffner, D. W. (2002). *Bacillus megaterium* spore germination is influenced by inoculum size. *Journal of Applied Microbiology*, 92(5):879–884.

Carlin, F., Broussolle, V., Perelle, S., Litman, S. & Fach, P. (2004). Prevalence of *Clostridium botulinum* in food raw materials used in repfeds manufactured in france. *International Journal of Food Microbiology*, 91(2):141–145.

Carlin, F., Fricker, M., Pielaat, A., Heisterkamp, S., Shaheen, R., Salonen, R., Svensson, B., Nguyen-The, C. & Ehling-Schulz, M. (2006). Emetic toxin-producing strains of *Bacillus cereus* show distinct characteristics within the bacillus cereus group. *International Journal of Food Microbiology*, 109(1):132–138.

Carlin, F., Girardin, H., Peck, M. W., Stringer, S. C., Barker, G. C., Martinez, A., Fernandez, A., Fernandez, P., Waites, W. M., Movahedi, S., van Leusden, F., Nauta, M., Moezelaar, R., Del Torre, M. & Litman, S. (2000a). Research on factors allowing a risk assessment of spore-forming pathogenic bacteria in cooked chilled foods containing vegetables: a FAIR collaborative project. *International Journal of Food Microbiology*, 60(2-3):117–135.

Carlin, F., Guinebretiere, M. H., Choma, C., Pasqualini, R., Braconnier, A. & Nguyen-the, C. (2000b). Spore-forming bacteria in commercial cooked, pasteurised and chilled vegetable purees. *Food Microbiology*, 17(2):153–165.

Carmody, R. N., Weintraub, G. S. & Wrangham, R. W. (2012). Reply to wollstonecroft et al.: Cooking increases the bioavailability of starch from diverse plant sources. *Proceedings of the National Academy of Sciences of the United States of America*, 109(17):E992–E992.

Cates, S. C., Moreales, R., Karns, S., Jaykus, L., Kosa, K., Teneyck, T., Moore, C. & Cowen, P. (2006). Consumer knowledge, storage, and handling practices regarding *Listeria* in frankfurters and deli meats: Results of a web-based survey. *Journal of Food Protection*, 69(7):1630–1639.

Cava-Roda, R., Taboada, A., Palop, A., Lopez-Gomez, A. & Marin-Iniesta, F. (2012). Heat resistance of *Listeria monocytogenes* in semi-skim milk supplemented with vanillin. *International Journal of Food Microbiology*, 157(2):314–318.

Cerf, O. (1977). Tailing of survival curves of bacterial spores. *Journal of Applied Bacteriology*, 42(1):1–19.

Ceuppens, S. (2012). *Growth characteristics, gene expression and toxin production or Bacillus cereus in the simulated intestine*. Ph.D. thesis, Faculty of Bioscience Engineering, Ghent University.

Ceuppens, S., Rajkovic, A., Heyndrickx, M., Tsilia, V., Van De Wiele, T., Boon, N. & Uyttendaele, M. (2011). Regulation of toxin production by *Bacillus cereus* and its food safety implications. *Critical Reviews in Microbiology*, 37(3):188–213.

Ceuppens, S., Uyttendaele, M., Drieskens, K., Rajkovic, A., Boon, N. & Van de Wiele, T. (2012). Survival of *Bacillus cereus* vegetative cells and spores during in vitro simulation of gastric passage. *Journal of Food Protection*, 75(4):690–694.

Choma, C., Clavel, T., Dominguez, H., Razafindramboa, N., Soumille, H., Nguyen-the, C. & Schmitt, P. (2000a). Effect of temperature on growth characteristics of *Bacillus cereus*TZ415. *International Journal of Food Microbiology*, 55(1-3):73–77.

Choma, C., Guinebretiere, M. H., Carlin, F., Schmitt, P., Velge, P., Granum, P. E. & Nguyen-The, C. (2000b). Prevalence, characterization and growth of *Bacillus cereus* in commercial cooked chilled foods containing vegetables. *Journal of Applied Microbiology*, 88(4):617–625.

Chorin, E., Thuault, D., Cleret, J. J. & Bourgeois, C. M. (1997). Modelling *Bacillus cereus* growth. *International Journal of Food Microbiology*, 38(2-3):229–234.

Ciarciaglini, G., Hill, P., Davies, K. W., McClure, P., Kilsby, D. C., Brown, M. & Coote, P. (2000). Germination-induced bioluminescence, a route to determine the inhibitory effect of as combination preservation treatment on bacterial spores. *Applied and Environmental Microbiology*, 66:3735–3742.

Clayton, D. & Griffith, C. J. (2003). An investigation of the factors underlying consumers' implementation of specific food safety practices. *British Food Journal*, 105(7):434–453.

Codex Alimentarius Commission (2007). Principles and guidelines for the conduct of microbiological risk management (MRM). Technical Report CAC/GL 63 - 2007, Food and Agriculture Organization of the United Nations/World Health Organization, Rome.

Codex Committee on Food Hygiene (CCFH) (1999). Code of hygienic practice for refrigerated packaged foods with extended shelf life. Technical Report CAC/RCP 46-(1999), Food and Agriculture Organization of the United Nations/World Health Organization, Rome.

Coleman, W., Chen, D., Li, Y., Cowan, A. & Setlow, P. (2007). How moist heat kills spores of *Bacillus subtilis*. *Journal of Bacteriology*, 189(23):8458–8466.

Coleman, W. & Setlow, P. (2009). Analysis of damage due to moist heat treatment of spore of *Bacillus subtilis*. *Journal of Applied Microbiology*, 106:1600–1607.

Coleman, W., Zhang, P., Li, Y. & Setlow, P. (2010). Mechanism of killing of spores of *Bacillus cereus* and *Bacillus megaterium* by wet heat. *Letters in Applied Microbiology*, 50:507–514.

Collado, J., Fernandez, A., Cunha, L. M., Ocio, M. J. & Martinez, A. (2003a). Improved model based on the weibull distribution to describe the combined effect of pH and temperature on the heat resistance of *Bacillus cereus* in carrot juice. *Journal of Food Protection*, 66(6):978–984.

Collado, J., Fernandez, A., Rodrigo, M., Camat, J. & Lopez, A. M. (2003b). Kinetics of deactivation of *Bacillus cereus* spores. *Food Microbiology*, 20(5):545–548.

Collado, J., Fernandez, A., Rodrigo, M. & Martinez, A. (2006). Modelling the effect of a heat shock and germinant concentration on spore germination of a wild strain of *Bacillus cereus*. *International Journal of Food Microbiology*, 106(1):85–89.

Cope, S., Frewer, L., Houghton, J., Rowe, G., Fischer, A. & de Jonge, J. (2010). Consumer perceptions of best practice in food risk communication and management: Implications for risk analysis policy. *Food Policy*, 35(4):349–357.

Coroller, L., Leguerinel, I. & Mafart, P. (2001). Effect of water activities of heating and recovery media on apparent heat resistance of *Bacillus cereus* spores. *Applied and Environmental Microbiology*, 67(1):317–322.

Costa, A. I. D., Schoolmeester, D., Dekker, M. & Jongen, W. M. F. (2007). To cook or not to cook: A means-end study of motives for choice of meal solutions. *Food Quality and Preference*, 18(1):77–88.

Couvert, O., Leguerinel, I. & Mafart, P. (1999). Modelling the overall effect of pH on the apparent heat resistance of *Bacillus cereus* spores. *International Journal of Food Microbiology*, 49(1-2):57–62.

Cowell, N. (1997). The first British patent for food preservation. *Food Science and technology today*, 11:225–228.

Crépet, A., Stahl, V. & Carlin, F. (2009). Development of a hierarchical bayesian model to estimate the growth parameters of *Listeria monocytogenes* in minimally processed fresh leafy salads. *International Journal of Food Microbiology*, 131(2-3):112–119.

Cronin, U. P. & Wilkinson, M. G. (2009). The growth, physiology and toxigenic potential of *Bacillus cereus* in cooked rice during storage temperature abuse. *Food Control*, 20(9):822–828.

Daelman, J., Aditya, S., Vermeulen, A., Uyttendaele, M., Devlieghere, F. & Membré, J. M. (2013a). Development of a time-to-detect growth model for heat-treated *Bacillus cereus* spores. *International Journal of Food Microbiology*, 165(3):231–240.

Daelman, J., Jacxsens, L., Devlieghere, F. & Uyttendaele, M. (2013b). Microbial safety and quality of various types of cooked chilled foods. *Food Control*, 30(2):510–517.

Daelman, J., Jacxsens, L., Lahou, E., Devlieghere, F. & Uyttendaele, M. (2013c). Assessment of the microbial safety and quality of cooked chilled foods and their production process. *International Journal of Food Microbiology*, 160(3):193–200.

Daelman, J., Jacxsens, L., Membré, J. M., Sas, B., Devlieghere, F. & Uyttendaele, M. (2013d). Behaviour of Belgian consumers, related to the consumption, storage and preparation of cooked chilled foods. *Food Control*, 34(2):681–690.

Daelman, J., Membré, J. M., Jacxsens, L., Vermeulen, A., Devlieghere, F. & Uyttendaele, M. (2013e). A Quantitative Microbiological Exposure Assessment model for *Bacillus cereus* in REPFEDs. *International Journal of Food Microbiology*, 166(23):433–449.

Daelman, J., Membré, J. M., Uyttendaele, M. & Devlieghere, F. (2012). A bayesian model for *Bacillus cereus* contamination in raw materials used for cooked chilled food production. In *Belgian-Dutch Conference on Machine Learning (Benelearn)*, p. 62. Ghent (Belgium).

Daelman, J., Vermeulen, A., Willemyns, T., Ongenaert, R., Jacxsens, L., Uyttendaele, M. & Devlieghere, F. (2013f). Growth no growth models for heat-treated psychrotrophic *Bacillus cereus* spores under cold storage. *International Journal of Food Microbiology*, 161(1):7–15.

de Sarrau, B., Clavel, T., Bornard, I. & Nguyen-The, C. (2013). Low temperatures and fermentative metabolism limit peptidoglycan digestion of *Bacillus cereus*. impact on colony forming unit counts. *Food Microbiology*, 33(2):213–220.

de Sarrau, B., Clavel, T., Clerte, C., Carlin, F., Ginies, C. & Christophe, N. T. (2012). Influence of anaerobiosis and low temperature on *Bacillus cereus* growth, metabolism, and membrane properties. *Applied and Environmental Microbiology*, 78(6):1715–1723.

De Standaard (2013). Kinderen van crèche in borgerhout werden onwel door voedselvergiftiging. Newspaper. URL `http://www.standaard.be/cnt/DMF20120903_168`. Consulted May 31, 2013.

De Vries, Y.P. (2006). *Bacillus cereus spore formation, structure, and germination*. Ph.D. thesis, Wageningen University, Wageningen, The Netherlands. 128 p.

De Vriese, S., De Backer, G., De Henauw, S., Huybrechts, I., Kortnitzer, K., Leveque, A., Moreau, M. & Van Oyen, H. (2005). The Belgian food consumption survey: aims, design an methods. *Archives of Public Health*, 63:1–16.

Debacker, N., Temme, L., Cox, B., Huybrechts, I. & Van Oyen, H. (2007). *De Belgische voedselconsumptiepeiling: Voedingsgewoonten van de Belgische bevolking ouder dan 15 jaar.* Wetenschappelijk instituut volksgezondheid, Brussels.

Del Torre, M., Della Corte, M. & Stecchini, M. L. (2001). Prevalence and behaviour of *Bacillus cereus* in a REPFED of italian origin. *International Journal of Food Microbiology*, 63(3):199–207.

Del Torre, M., Stecchini, M. L., Braconnier, A. & Peck, M. W. (2004). Prevalence of clostridium species and behaviour of *Clostridium botulinum* in gnocchi, a REPFED of italian origin. *International Journal of Food Microbiology*, 96(2):115–131.

Delignette-Muller, M. L., Cornu, M., Pouillot, R. & Denis, J. B. (2006). Use of bayesian modelling in risk assessment: Application to growth of *Listeria monocytogenes* and food flora in cold-smoked salmon. *International Journal of Food Microbiology*, 106(2):195–208.

Delignette-Muller, M. L., Pouillot, R., Denis, J. B. & Dutang, C. (2010). *Fitdistrplus*: Help to fit of a parametric distribution to non-censored or censored data. http://cran.r-project.org/web/packages/fitdistrplus/fitdistrplus.pdf.

Delignette-Muller, M. L. & Rosso, L. (2000). Biological variability and exposure assessment. *International Journal of Food Microbiology*, 58(3):203–212.

den Aantrekker, E. D., Boom, R. M., Zwietering, M. H. & van Schothorst, M. (2003). Quantifying recontamination through factory environments - a review. *International Journal of Food Microbiology*, 80(2):117–130.

den Besten, H. M. W., Mataragas, M., Moezelaar, R., Abee, T. & Zwietering, M. H. (2006). Quantification of the effects of salt stress and physiological state on thermotolerance of *Bacillus cereus* ATCC 10987 and ATCC 14579. *Applied and Environmental Microbiology*, 72(9):5884–5894.

Derens, E., Guilpart, J., Palagos, B. & Prosen, E. (2004). Données chiffrées sur la chaîne du froid des produits réfrigérés. *Revue Générale du Froid*, 1045:27–32.

DG Sanco (2013). Eu databaes on food additives. Online. URL `https://webgate.ec.europa.eu/sanco_foods/`. Consulted June 1, 2013.

Dierick, K., Van Coillie, E., Swiecicka, I., Meyfroidt, G., Devlieger, H., Meulemans, A., Hoedemaekers, G., Fourie, L., Heyndrickx, M. & Mahillon, J. (2005). Fatal family outbreak

of *Bacillus cereus*-associated food poisoning. *Journal of Clinical Microbiology*, 43(8):4277–4279.

Domenech, E., Amoros, J. A., Martorell, S. & Escriche, I. (2012). Safety assessment of smoked fish related to *Listeria monocytogenes* prevalence using risk management metrics. *Food Control*, 25(1):233–238.

Domenech, E., Escriche, I. & Martorell, S. (2010). Exposure assessment based on a combination of event and fault tree analyses and predictive modelling. *Food Control*, 21(10):1338–1348.

Dufrenne, J., Soentoro, P., Tatini, S., Day, T. & commentrmans, S. (1994). Characteristics of *Bacillus cereus* related to safe food-production. *International Journal of Food Microbiology*, 23(1):99–109.

Duport, C., Thomassin, S., Bourel, G. & Schmitt, P. (2004). Anaerobiosis and low specific growth rates enhance hemolysin BL production by *Bacillus cereus*F4430/73. *Archives of Microbiology*, 182(1):90–95.

ECFF (2006). Recommendations for the production of prepackaged chilled food, 2nd edition. Technical report, European Chilled Food Federation.

EFSA (2005a). Opinion of the scientific panel on biological hazards on *Bacillus cereus* and other *Bacillus* spp in foodstuffs. *The EFSA Journal*, 175:1–48.

EFSA (2005b). Opinion of the scientific panel on biological hazards on the request from the commission related to *Clostridium* spp in foodstuffs.1. *The EFSA Journal*, 199:1–65.

EFSA (2007). Request for updating the former scvph opinion on *Listeria monocytogenes* risk related to ready-to-eat foods and scientific advice on different levels of *Listeria monocytogenes* in ready-to-eat foods and the related risk for human illness. *The EFSA Journal*, 599:1–42.

Ehling-Schulz, M., Svensson, B., Guinebretiere, M. H., Lindback, T., Andersson, M., Schulz, A., Fricker, M., Christiansson, A., Granum, P. E., Martlbauer, E., Nguyen-The, C., Salkinoja-Salonen, M. & Scherer, S. (2005). Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. *Microbiology-Sgm*, 151:183–197.

Ekman, J., Kruglov, A., Andersson, M., Mikkola, R., Raulio, M. & Salkinoja-Salonen, M. (2012). Cereulide produced by *Bacillus cereus* increases the fitness of the producer organism in low-potassium environments. *Microbiology*, 145:1106–1116.

Enright, J. B., Sadler, W. W. & Thomas, R. C. (1957). Pasteurization of milk containing the organism of Q-fever. *Am.J Public Health Nations.Health*, 47(6):695–700.

Ergönül, B. (2013). Consumer awareness and perception to food safety: A consumer analysis. *Food Control*, 32:461–471.

Esty, J. R. & Meyer, R. F. (1922). The heat resistance of the spores of *B. botulinus* and allied anaerobes. *Journal of Infectious Disease*, 31:650.

EU CRL for *Listeria monocytogenes* (2008). Technical guidance document on shelf-life studies for *Listeria monocytogenes* in ready-to-eat foods. URL `http://ec.europa.eu/food/food/biosafety/salmonella/docs/shelflife_listeria_monocytogenes_en.pdf`.

European Food Safety Authority, European Centre for Disease Prevention and Control (2011). The European union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2009. *EFSA Journal*, 9(3):1–378.

European Food Safety Authority, European Centre for Disease Prevention and Control (2012). The European union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. *EFSA Journal*, 10(3):2597.

European Food Safety Authority, European Centre for Disease Prevention and Control (2013). The European union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011. *EFSA Journal*, 11(4):3129.

Evans, J., Stanton, S. J.I. an Russel & James, S. (1991). *Consumer Handling of Chilled Foods: A Survey of Time and Temperature Conditions*. Ministry of Agriculture, Fisheries and Food, Bristol, UK.

Faille, C., Lebret, V., Gavini, F. & Maingonnat, J. F. (1997). Injury and lethality of heat treatment of *Bacillus cereus* spores suspended in buffer and in poultry meat. *Journal of Food Protection*, 60(5):544–547.

Farber, J. M. & Peterkin, P. I. (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Reviews*, 55(3):476–511.

Farber, J. M., Sanders, G., Speirs, J., D'Aoust, J., Emmons, D. & McKellar, R. C. (1988). Thermal resistance of *Listeria monocytogenes* in inoculated and naturally contaminated raw milk. *International Journal of Food Microbiology*, 7(4):277–289.

FASFC (2006). Trends and sources report on zoonotic agents in Belgium in 2006. Technical report, Federal Agency for the Safety of the Food Chain.

FDA (2000). Gras notice for specified uses of nisin. URL `http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn0065.pdf`. Consulted June 1,2013.

Fermanian, C., Fremy, J. M. & Claisse, M. (1994). Effect of temperature on the vegetative growth of type and field strains of *Bacillus cereus*. *Letters in Applied Microbiology*, 19(6):414–418.

Fernandez, A., Collado, J., Cunha, L. M., Ocio, M. J. & Martinez, A. (2002). Empirical model building based on weibull distribution to describe the joint effect of pH and temperature on the thermal resistance of *Bacillus cereus* in vegetable substrate. *International Journal of Food Microbiology*, 77(1-2):147–153.

Fernandez, A., Ocio, M. J., Fernandez, P., Rodrigo, M. & Martinez, A. (1999). Application of nonlinear regression analysis to the estimation of kinetic parameters for two enterotoxigenic strains of *Bacillus cereus* spores. *Food Microbiology*, 16(6):607–613.

Fernandez, A., Ocio, M. J., Fernandez, P. S. & Martinez, A. (2001a). Effect of heat activation and inactivation conditions on germination and thermal resistance parameters of *Bacillus cereus* spores. *International Journal of Food Microbiology*, 63(3):257–264.

Fernandez, P. S., Baranyi, J. & Peck, M. W. (2001b). A predictive model of growth from spores of non-proteolytic *Clostridium botulinum* in the presence of different CO_2 concentrations as influenced by chill temperature, pH and NaCl. *Food Microbiology*, 18(4):453–461.

Finlay, W. J. J., Logan, N. A. & Sutherland, A. D. (2000). *Bacillus cereus* produces most emetic toxin at lower temperatures. *Letters in Applied Microbiology*, 31(5):385–389.

Finlay, W. J. J., Logan, N. A. & Sutherland, A. D. (2002). *Bacillus cereus* emetic toxin production in cooked rice. *Food Microbiology*, 19(5):431–439.

Fisher, A., De Jonge, A., De Jonge, R., Frewer, L. & Nauta, M. (2005). Improving food safety in the domestic environment: the need for a transdisciplinary approach. *Risk Analysis*, 25(3):503–517.

FPS Economy, SMEs, Self-employed and Energy (2011). Structuur van de belgische bevolking volgens leeftijd en geslacht.

Frewer, L. (2004). The public and effective risk communication. *Toxicology Letters*, 149:391–397.

From, C., Pukall, R., Schumann, P., Hormazabal, V. & Granum, P. E. (2005). Toxin-producing ability among *Bacillus* spp. outside the *Bacillus cereus* group. *Applied and Environmental Microbiology*, 71(3):1178–83.

Fryer, P. J. & Robbins, P. T. (2005). Heat transfer in food processing: ensuring product quality and safety. *Applied Thermal Engineering*, 25(16):2499–2510.

Gaillard, S., Leguerinel, I. & Mafart, P. (1998). Model for combined effects of temperature, pH and water activity on thermal inactivation of *Bacillus cereus* spores. *Journal of Food Science*, 63(5):887–889.

Gaillard, S., Leguerinel, I., Savy, N. & Mafart, P. (2005). Quantifying the combined effects of the heating time, the temperature and the recovery medium pH on the regrowth lag time of *Bacillus cereus* spores after a heat treatment. *International Journal of Food Microbiology*, 105(1):53–58.

Gao, Y.-L. & Ju, X.-R. (2010). Modelling the effects of food ingredients and pH on high-pressure processing inactivation of *Bacillus cereus* spores: a laboratorial study. *International Journal of Food Science and Technology*, 45:1862–1869.

Garrido, V., Garcia-Jalon, I., Vitas, A. I. & Sanaa, M. (2010). Listeriosis risk assessment: Simulation modelling and "what if" scenarios applied to consumption of ready-to-eat products in a spanish population. *Food Control*, 21(3):231–239.

Gaze, J. (2006). *Pasteurisation: A Food Industry Practical Guide (Second Edition), Guideline No. 51*. Campden & Chorleywood Food Research Association, Chipping Camden.

Gaze, J. & Brown, G. (1990). Determination of the heat resistance of a strain of non-proteolytic *Clostridium botulinum* type B and a strain of type E, heated in cod and carrot homogenate over the temperature range 70 to 90°C. Technical report, Campden & Chorleywood Food Research Association.

Gaze, J. E., Brown, G., Gaskell, D. E. & Banks, J. G. (1989). Heat resistance of *Listeria monocytogenes* in homogenates of chicken, beef steak and carrot. *Food Microbiology*, 6(4):251–259.

Geeraerd, A., Valdramidis, V. P. & Van Impe, J. (2005). Ginafit, a freeware tool to assess non-log-linear microbial survivor curves. *International Journal of Food Microbiology*, 102:95–105.

Ghosh, S. & Setlow, P. (2009a). Isolation and characterization of superdormant spores of *Bacillus* species. *Journal of Bacteriology*, 191(6):1787–1797.

Ghosh, S. & Setlow, P. (2009b). The preparation, germination properties and stability of superdormant spores of *Bacillus cereus*. *Journal of Applied Microbiology*, 108:582–590.

Ghosh, S., Zhang, P., Li, Y. & Setlow, P. (2009). Superdormant spores of *Bacillus* species have elevated wet-heat resistance and temperature requirements for heat activation. *Journal of Bacteriology*, 191(18):5584–5591.

Gibbs, P. (2002). Characteristics of spore-forming bacteria. In Blackburn, C. D. W. & McClure, P., editors, *Foodborne pathogens*. Woodhead Publishin Ltd and CRC pres LLC.

Gill, D. M. (1982). Bacterial toxins: A table of lethal amounts. *Microbiological Reviews*, 46 (1):86–94.

Gofton, L. (1995). Convenience and the moral status of consumer practices. In Marshal, D., editor, *Food choice and the consumer*. Blackie Academic and Professional.

Goldwin, S. & Coppings, R. (2005). Analysis of consumer food-handling practices from grocer to home including transport and storage of selected foods. *Journal of Food Distribution Research*, 36:55–62.

Gonzalez, I., Lopez, M., Martinez, S., Bernardo, A. & Gonzalez, J. (1999). Thermal inactivation of *Bacillus cereus* spores formed at different temperatures. *International Journal of Food Microbiology*, 51(1):81–84.

Gonzalez, I., Lopez, M., Mazas, M., Gonzalez, J. & Bernardo, A. (1997). Thermal resistance of *Bacillus cereus* spores as affected by additives in the recovery medium. *Journal of Food Safety*, 17(1):1–12.

Gorris, L. G. M. (2005). Food safety objective: An integral part of food chain management. *Food Control*, 16(9):801–809.

Gould, G. W. (1999). Sous vide foods: conclusion of an ECFF botulinum working party. *Food Control*, 10:47–51.

Gould, G. W. (2006). History of science–spores. *Journal of Applied Bacteriology*, 101(3):507–513.

Granum, P. E. (1997). *Bacillus cereus*. In Doyle, M., Beuchat, L. & Montville, T., editors, *Fundamentals in Food Microbiology*, pp. 327–336. ASM Press Washington, DC.

Granum, P. E. & Lund, T. (1997). *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Letters*, 157:223–228.

Guinebretière, M. H., Broussolle, V. & Nguyen-The, C. (2002). Enterotoxigenic profiles of food-poisoning and food-borne *Bacillus cereus* strains. *Journal of Clinical Microbiology*, 40(8):3053–3056.

Guinebretière, M. H., Girardin, H., Dargaignaratz, C., Carlin, F. & Nguyen-The, C. (2003). Contamination flows of *Bacillus cereus* and spore-forming aerobic bacteria in a cooked, pasteurized and chilled zucchini puree processing line. *International Journal of Food Microbiology*, 82(3):223–232.

Guinebretière, M. H. & Nguyen-The, C. (2003). Sources of *Bacillus cereus* contamination in a pasteurized zucchini puree processing line, differentiated by two PCR-based methods. *Fems Microbiology Ecology*, 43(2):207–215.

Guinebretière, M. H., Thompson, F., Sorokin, A., Normand, P., Dawyndt, P., Ehling-Schulz, M., Svensson, B., Sanchis, V., Nguyen-The, C., Heyndrickx, M. & P., D. V. (2008). Ecological diversification in the *Bacillus cereus* group. *Environmental Microbiology*, 10(4):851–865.

Hansen, T. B. & Knochel, S. (2001). Factors influencing resuscitation and growth of heat injured *Listeria monocytogenes* 13-249 in sous vide cooked beefs. *International Journal of Food Microbiology*, 63(1-2):135–147.

Havelaar, A. H., Nauta, M. J. & Jansen, J. T. (2004). Fine-tuning food safety objectives and risk assessment. *International Journal of Food Microbiology*, 93(1):11–29.

Heo, S. K., Lee, J. Y., Baek, S. B. & Ha, S. D. (2009). A response surface model to describe the effect of temperature and pH on the growth of *Bacillus cereus* in cooked rice. *Journal of Food Protection*, 72(6):1296–1300.

Hoa, N., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P., Ammendola, S., Ricca, E. & Cutting, Z. (2000). Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. *Applied and Environmental Microbiology*, 66:5241–5247.

Holdsworth, S. (2004). Optimising the safety and quality of thermally processed packaged foods. In Richardson, P., editor, *Improving the thermal processing of foods*. Woodhead publishing Ltd.

Holsinger, V., Smith, P., Smith, J. & Palumbo, S. (1992). Thermal destruction of *Listeria monocytogenes* in ice cream mix. *Journal of Food Protection*, 55:234–237.

Hornstra, L. M., van der Voort, M., Wijnands, L. M., Roubos-van den Hil, P. J. & Abee, T. (2009). Role of germinant receptors in Caco-2 cell-initiated germination of *Bacillus cereus*ATCC 14579 endospores. *Applied and Environmental Microbiology*, 75(4):1201–1203.

Hoton, F. M., Fornelos, N., N'Guessan, E., Hu, X. M., Swiecicka, I., Dierick, K., Jaaskelainen, E., Salkinoja-Salonen, M. & Mahillon, J. (2009). Family portrait of *Bacillus cereus* and bacillus weihenstephanensis cereulide-producing strains. *Environmental Microbiology Reports*, 1(3):177–183.

ICMSF (1996). *Microorganisms in Foods 5: Microbiological Specifications of Food Pathogens*. Blackie Academic and Professional, London.

ICMSF (2002). *Microorganisms in Foods 7. Microbiological testing in food safety management*. Kluwer Academic / Plenum Publishers, New York, USA.

ICMSF (2005). *Microorganisms in Foods 6 (second edition). Microbial ecology of food commodities.* Kluwer Academic/ Plenum Publishers, New York.

ICMSF (2006). A simplified guide to understanding and using food safety objectives and performance objectives.

ISO (1999). Water quality – enumeration of culturable microorganisms – colony count by inoculation in a nutrient agar culture medium (ISO 6222:1999).

ISO (2004). Microbiology of food and animal feeding stuffs – horizontal method for the enumeration of presumptive *Bacillus cereus*– colony-count technique at 30 degrees C (ISO 7932:2004).

Jabs, J. & Devine, C. M. (2006). Time scarcity and food choices: An overview. *Appetite*, 47(2):196–204.

Jaloustre, S., Cornu, M., Morelli, E., Noël, V. & Delignette-Muller, M. L. (2011). Bayesian modeling of *Clostridium perfringens* growth in beef-in-sauce products. *Food Microbiology*, 28:311–320.

James, S. R. (1989). Hominid use of fire in the lower and middle pleistocene - a review of the evidence. *Current Anthropology*, 30(1):1–26.

Jay, J. M. (1996). Prevalence of *Listeria* spp in meat and poultry products. *Food Control*, 7(4-5):209–214.

Jevsnik, M., Hlebec, V. & Raspor, P. (2008). Consumers' awareness of food safety from shopping to eating. *Food Control*, 19:737–745.

Johnson, A., Donkin, A., Morgan, K., Lilley, J., Neale, R., Page, R. & Silburn, R. (1998). Food safety knowledge and practice among elderly people living at home. *Journal of Epidemiology and Community Health*, 52:745–745.

Johnson, J. L., Doyle, M. P. & Cassens, R. G. (1990). *Listeria monocytogenes* and other listeria spp in meat and meat-products - a review. *Journal of Food Protection*, 53(1):81–91.

Juliao, P., Maslanka, S., Dykes, J., Gaul, L., Bagdure, S., Granzow-Kibiger, L., Salehi, E., Zink, D., Neligan, R., Barton-Behravesh, C., Luquez, C., Biggerstaff, M., Lynch, M., Olson, C., Williams, I. & Barzilay, E. (2013). National outbreak of type a foodborne botulism associated with a widely distributed commercially canned hot dog chili sauce. *Clinical Infectious Diseases*, 56(3):376–381.

Juneja, V. K. & Eblen, B. S. (2000). Heat inactivation of *Salmonella typhimurium* dt104 in beef as affected by fat content. *Letters in Applied Microbiology*, 30:461–467.

Juneja, V. K., Eblen, B. S., Marmer, B. S., Williams, A. C., Palumbo, S. & Miller, A. J. (1995). Thermal-resistance of nonproteolytic type-B and type-E *Clostridium botulinum* spores in phosphate buffer and turkey slurry. *Journal of Food Protection*, 58(7):758–763.

Katzin, L., Sandholzer, L. & Strong, M. (1943). Application of the decimal reduction time principle to a study of the resistance of coliform bacteria to pasteurization. *Journal of Bacteriology*, 45 (3):265–272.

Kennedy, J., Jackson, V., Blair, I. S., McDowell, D. A., Cowan, C. & Bolton, D. J. (2005). Food safety knowledge of consumers and the microbiological and temperature status of their refrigerators. *Journal of Food Protection*, 68(7):1421–1430.

Kim, H., Lee, N., Lee, D., Hong, W., Lee, S., Kim, C. & Paik, H. (2008). Improvement of microbiological safety of sous vide processed soybean sprouts: Nisin and *Bacillus cereus* challenge. *Food Science and Biotechnology*, 17(1):166–171.

Kim, S., Shin, S., Song, C., Jo, E., Kim, H. & Park, J. (2009). Destruction of *Bacillus licheniformis* spores by microwave irradiation. *Journal of Applied Microbiology*, 106(3):877–558.

Kim, S., Yun, S., Lee, S., Hwang, I. & Rhee, M. (2013). Temperature increase of foods in car trunk and the potential hazard for microbial growth. *Food Control*, 29:66–70.

Klein, G. (2011). Molecular characterization of the probiotic strain *Bacillus cereus* var. toyoi ncimb 40112 and differentiation from food poisoning strains. *Journal of Food Protection*, 74(7):1189–1193.

Konuma, H., Shinagawa, K., Tokumaru, M., Onoue, Y., Konno, S., Fujino, N., Shigehisa, T., Kurata, H., Kuwabara, Y. & Lopes, C. A. M. (1988). Occurrence of *Bacillus cereus* in meat-products, raw meat and meat product additives. *Journal of Food Protection*, 51(4):324–326.

Kotiranta, A., Lounatmaa, K. & Haapasalo, M. (2000). Epidemiology and pathogenesis of *Bacillus cereus* infections. *Microbes and Infection*, 2(2):189–198.

Koutsoumanis, K. (2001). Predictive modeling of the shelf life of fish under nonisothermal conditions. *Applied and Environmental Microbiology*, 67(4):1821–1829.

Lahou, E., Jacxsens, L., Daelman, J., Van Landeghem, F. & Uyttendaele, M. (2012). Microbiological performance of a food safety management system in a food service operation. *Journal of Food Protection*, 75(4):706–716.

Lanciotti, R., Sinigaglia, M., Gardini, F., Vannini, L. & Guerzoni, M. E. (2001). Growth/no growth interfaces of *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella enteritidis* in model systems based on water activity, pH, temperature and ethanol concentration. *Food Microbiology*, 18(6):659–668.

Langeveld, L., Van Spronsen, W. A., Van Beresteijn, E. & Notermans, S. H. W. (1996). Consumption by healthy adults of pasteurized milk with a high concentration of *Bacillus cereus*: A double-blind study. *Journal of Food P*, 59(7):723–726.

Lasta, J. A., Rodriguez, R., Zanelli, M. & Margaria, C. A. (1992). Bacterial count from bovine carcasses as an indicator of hygiene at slaughtering places - a proposal for sampling. *Journal of Food Protection*, 55(4):271–278.

Laurent, Y., Arino, S. & Rosso, L. (1999). A quantitative approach for studying the effect of heat treatment conditions on resistance and recovery of *Bacillus cereus* spores. *International Journal of Food Microbiology*, 48(2):149–157.

Leguerinel, I., Couvert, O. & Mafart, P. (2007). Modelling the influence of the sporulation temperature upon the bacterial spore heat resistance, application to heating process calculation. *International Journal of Food Microbiology*, 114(1):100–104.

Leguerinel, I. & Mafart, P. (2001). Modelling the influence of pH and organic acid types on thermal inactivation of *Bacillus cereus* spores. *International Journal of Food Microbiology*, 63(1-2):29–34.

Leistner, L. & Gorris, L. G. M. (1995). Food preservation by hurdle technolo. *Trends in Food Science & Technology*, 6:41–46.

Lekogo, B., Coroller, L., Mathot, A., Mafart, P. & Leguerinel, I. (2010). Modelling the influence of palmitic, palmitoleic, stearic and oleic acids on apparent heat resistance of spores of *Bacillus cereus* NTCC 11145 and *Clostridium sporogenes* pasteur 79.3. *International Journal of Food Microbiology*, 141(3):242–247.

Lianou, A. & Sofos, J. N. (2007). A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. *Journal of Food Protection*, 70(9):2172–2198.

Lo Nostro, A., Pesavento, G., Ducci, B., Nieri, D. & Comodo, N. (2010). Prevalence and antibiotic susceptibility of *Listeria* spp. isolated from raw meat and retail foods. *Food Control*, 21(5):708–713.

Loureiro, M. (2008). Liability and food safety provision: Empirical evidence from the US. *International Review of Law and Economics*, 28(3):204–211.

Lovdal, I., Hovda, M., Granum, P. E. & Rosnes, J. T. (2011). Promoting *Bacillus cereus* spore germination for subsequent inactivation by mild heat treatment. *Journal of Food Protection*, 74(12):2079–2089.

Luber, P., Brynestad, S., Topsch, D., Scherer, K. & Bartelt, E. (2006). Quantification of *Campylobacter* species cross-contamination during handling of contaminated fresh chicken parts in kitchens. *Applied and Environmental Microbiology*, 72(1):66–70.

Lund, B. M. (1993). Quantification of factors affecting the probability of development of pathogenic bacteria, in particular *Clostridium botulinum*, in foods. *Journal of Industrial Microbiology*, 12(3-5):144–155.

Lund, B. M. & Peck, M. W. (1994). Heat resistance and recovery of spores of non-proteolytic *Clostridium botulinum* in relation to refrigerated, processed foods with and extended shelf life. *Journal of Applied Bacteriology Symposium Supplement*, 76:115S–128S.

Lund, T., De Buyser, M. & Granum, P. E. (2000). A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Molecular Biology*, 38(2):254–261.

Lunden, J. M., Autio, T. J. & Korkeala, H. J. (2002). Transfer of persistent *Listeria monocytogenes* contamination between food-processing plants associated with a dicing machine. *Journal of Food Protection*, 65(7):1129–1133.

Luning, P. A., Bango, L., Kussaga, J., Rovira, J. & Marcelis, W. J. (2008). Comprehensive analysis and differentiated assessment of food safety control systems: a diagnostic instrument. *Trends in Food Science & Technology*, 19(10):522–534.

Luning, P. A., Jacxsens, L., Rovira, J., Oses, S. M., Uyttendaele, M. & Marcelis, W. J. (2011). A concurrent diagnosis of microbiological food safety output and food safety management system performance: Cases from meat processing industries. *Food Control*, 22(3-4):555–565.

Lyytikainen, O., Autio, T., Maijala, R., Ruutu, P., Honkanen-Buzalski, T., Miettinen, M., Hatakka, M., Mikkola, J., Anttila, V. J., Johansson, T., Rantala, L., Aalto, T., Korkeala, H. & Siitonen, A. (2000). An outbreak of *Listeria monocytogenes* serotype 3a infections from butter in finland. *Journal of Infectious Diseases*, 181(5):1838–1841.

Mackey, B. M., Boogard, E., Hayes, C. M. & Baranyi, J. (1994). Recovery of heat-injured *Listeria monocytogenes*. *International Journal of Food Microbiology*, 22(4):227–237.

Mackey, B. M. & Bratchell, N. (1989). The heat-resistance of *Listeria monocytogenes*. *Letters in Applied Microbiology*, 9(3):89–94.

Mahler, H., Pasi, A., Kramer, J., Schulte, P., Scoging, A., Bar, W. & Krahenbuhl, S. (1997). Fulminant liver failuer in association with the emetic toxin of *Bacillus cereus*. *New England Journal of Medicine*, 336(16):1142–1148.

Malakar, P. K., Barker, G. C. & Peck, M. W. (2004). Modeling the prevalence of *Bacillus cereus* spores during the production of a cooked chilled vegetable product. *Journal of Food Protection*, 67(5):939–946.

Marin, J.-M. & Robert, C. (2007). *Bayesian Core: A practical approach to computational Bayesian statistics.* Springer, New York.

Markland, S., Farkas, D., Kniel, K. & Hoover, D. (2013). Pathogenic psychrotolerant spore-formers: an emerging challenge for low-temperature storage of minimally processed foods. *Foodborne Pathogens and Disease*, 10(5):413–419.

Martinez, S., Borrajo, R., Franco, I. & Carballo, J. (2007). Effect of environmental parameters on growth kinetics of *Bacillus cereus* (ATCC 7004) after mild heat treatment. *International Journal of Food Microbiology*, 117(2):223–227.

Membré, J. M. (2009). Practical application of predictive modelling techniques in a food business. In Heggum, C., editor, *Proceedings of International Symposium on Revolution in Food Safety Management,*, International Dairy Federation Bulletin, pp. 70–73.

Membré, J. M. (2012). Setting of thermal processes in a context of food safety objectives (FSOs) and related concepts. In Valdramidis, V. & Van Impe, J., editors, *Progress on Quantitative Approaches of Thermal Food Processing*. Nova Science Publishers, New York.

Membré, J. M., Kan-King-Yu, D. & Blackburn, C. D. (2008). Use of sensitivity analysis to aid interpretation of a probabilistic *Bacillus cereus* spore lag time model applied to heat-treated chilled foods (repfeds). *International Journal of Food Microbiology*, 128(1):28–33.

Membré, J. M. & Lambert, R. J. W. (2008). Application of predictive modelling techniques in industry: From food design up to risk assessment. *International Journal of Food Microbiology*, 128(1):10–15.

Membré, J. M., Laroche, M. & Magras, C. (2011). Assessment of levels of bacterial contamination of large wild game meat in europe. *Food Microbiology*, 28:1072–1079.

Membré, J. M., Leporq, B., Vialette, M., Mettler, E., Perrier, L., Thuault, D. & Zwietering, M. (2005). Temperature effect on bacterial growth rate: quantitative microbiology approach including cardinal values and variability estimates to perform growth simulations on/in food. *International Journal of Food*, 100:179–186.

Membré, J.-M. & van Zuijlen, A. (2011). A probabilistic approach to determine thermal process setting parameters: Application for commercial sterility of products. *International Journal of Food Microbiology*, 144(3):413–420.

Membré, J. M., Wemmenhove, E. & McClure, P. (2009). Exposure assessment model to combine thermal inactivation (log reduction) and thermal injury (heat-treated spore lag time) effects on non-proteolytic *Clostridium botulinum*. *Safety, Reliability and Risk Analysis: Theory, Methods and Applications*, 1-4:2295–2303.

Mikkola, R., Saris, N., Grigoriev, P., Andersson, M. & Salkinoja-Salonen, M. (1999). Ionophoretic properties and mitochondrial effects of cereulide - the emetic toxin of *Bacillus cereus*. *European Journal of Biochemistry*, 263:112–117.

Mols, M., van Kranenburg, R., Tempelaars, M. H., van Schaik, W., Moezelaar, R. & Abee, T. (2010). Comparative analysis of transcriptional and physiological responses of *Bacillus cereus*

to organic and inorganic acid shocks. *International Journal of Food Microbiology*, 137(1):13–21.

Mossel, D. A. & Struijk, C. B. (1991). Public health implication of refrigerated pasteurized ('sous-vide') foods. *International Journal of Food Microbiology*, 13(3):187–206.

Naranjo, M., Denayer, S., Botteldoorn, N., Delbrassinne, L., Veys, J., Waegenaere, J., Sirtaine, N., Driesen, R. B., Sipido, K. R., Mahillon, J. & Dierick, K. (2011). Sudden death of a young adult associated with *Bacillus cereus* food poisoning. *Journal of Clinical Microbiology*, 49(12):4379–4381.

National Advisory Committee on Microbiological Criteria For Foods (NACMCF) (2005). Considerations for establishing safety-based consume-by date labels for refrigerated ready-to-eat foods. *Journal of Food Protection*, 68(8):1761–1775.

Nauta, M. (2008). The modular process risk model (MPRM): a structured approach for food chain exposure assessment. In Schaffner, D., editor, *Microbial Risk Analysis of Foods*, pp. 99–136. ASM Press, Washington, DC.

Nauta, M. J. (2001). A modular process risk model structure for quantitative microbiological risk assessment and its application in an exposure assessment of *Bacillus cereus* in a repfed. Technical Report 149106 007, RIVM report 149106 007.

Nauta, M. J. (2002). Modelling bacterial growth in quantitative microbiological risk assessment: is it possible? *International Journal of Food Microbiology*, 73(2-3):297–304.

Nauta, M. J. (2005). Microbiological risk assessment models for partitioning and mixing during food handling. *International Journal of Food Microbiology*, 100(1-3):311–322.

Nauta, M. J., Litman, S., Barker, G. C. & Carlin, F. (2003). A retail and consumer phase model for exposure assessment of *Bacillus cereus*. *International Journal of Food Microbiology*, 83(2):205–218.

Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. & Setlow, P. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extrater- restrial environments. *Microbiology and Molecular Biology Reviews*, 64(3):548–572.

Nissen, H., Rosnes, J. T., Brendehaug, J. & Kleiberg, G. H. (2002). Safety evaluation of sous vide-processed ready meals. *Letters in Applied Microbiology*, 35(5):433–438.

Notermans, S., Dufrenne, J., Teunis, P., Beumer, R., Giffel, M. T. & Weem, P. P. (1997). A risk assessment study of *Bacillus cereus* present in pasteurized milk. *Food Microbiology*, 14(2):143–151.

Nyachuba, D. & Donnelly, C. (2005). Sublethal injury, pathogen virulence and adaptation. In Griffiths, M., editor, *Understanding pahtogen behaviour, virulence, stress response and resistance*. Woodhead Publishin Ltd, Cambridge, England.

Ochiai, Y., Yamada, F., Batmunkh, O., Mochizuki, M., Takano, T., Hondo, R. & Ueda, F. (2010). Prevalence of *Listeria monocytogenes* in retailed meat in the tokyo metropolitan area. *Journal of Food Protection*, 73(9):1688–1693.

Olmez, H. K. & Aran, N. (2005). Modeling the growth kinetics of *Bacillus cereus* as a function of temperature, pH, sodium lactate and sodium chloride concentrations. *International Journal of Food Microbiology*, 98(2):135–143.

Pal, A., Labuza, T. & Diez-Gonzalez, F. (2008). Evaluating the growth of *Listeria monocytogenes* in refrigerated ready-to-eat frankfurters: Influence of strain, temperature, packaging, lactate and diacetate, and background microflora. *Journal of Food Protection*, 71(9):1806–1816.

Panagou, E. Z. & Nychas, G. J. E. (2008). Dynamic modeling of *Listeria monocytogenes* growth in pasteurized vanilla cream after postprocessing contamination. *Journal of Food Protection*, 71(9):1828–1834.

Peck, M. W. (1997). *Clostridium botulinum* and the safety of refrigerated processed foods of extended durability. *Trends in Food Science & Technology*, 8(6):186–192.

Peck, M. W. & Fernandez, P. S. (1995). Effect of lysozyme concentration, heating at 90 degrees C, and then incubation at chilled temperatures on growth from spores of nonproteolytic *Clostridium botulinum*. *Letters in Applied Microbiology*, 21(1):50–54.

Peck, M. W., Goodburn, K. E., Betts, R. P. & Stringer, S. C. (2006). *Clostridium botulinum* in vacuum packed (VP) and modified atmosphere packed (MAP) chilled foods. Technical Report Project B13006, Institute of Food Research, Norwich, UK.

Peck, M. W., Goodburn, K. E., Betts, R. P. & Stringer, S. C. (2008). Assessment of the potential for growth and neurotoxin formation by non-proteolytic *Clostridium botulinum* in short shelf-life commercial foods designed to be stored chilled. *Trends in Food Science & Technology*, 19(4):207–216.

Peck, M. W., Lund, B. M., Fairbairn, D. A., Kaspersson, A. S. & Undeland, P. C. (1995). Effect of heat-treatment on survival of, and growth from, spores of nonproteolytic *Clostridium botulinum* at refrigeration temperatures. *Applied and Environmental Microbiology*, 61(5):1780–1785.

Peck, M. W. & Stringer, S. C. (2005). The safety of pasteurised in-pack chilled meat products with respect to the foodborne botulism hazard. *Meat Science*, 70(3):461–475.

Peleg, M. & Corradini, M. G. (2011). Microbial growth curves: What the models tell us and what they cannot. *Critical Reviews in Food Science and Nutrition*, 51(10):917–945.

Periago, P. M., Fernandez, P. S., Salmeron, M. C. & Martinez, A. (1998). Predictive model to describe the combined effect of pH and NaCl on apparent heat resistance of *Bacillus stearothermophilus*. *International Journal of Food Microbiology*, 44(1-2):21–30.

Petty, A., Melanson, K. & Greene, G. (2013). Self-reported eating rate aligns with laboratory measured eating rate but not with free-living meals. *Appetite*, 63:36–41.

Pina-Perez, M., SIlva-Angula, A., Rodrigo, D. & Lopez, A. M. (2012). A preliminary exposure assessment model for *Bacillus cereus* cells in a milk based beverage: Evaluating high pressure processing and antimicrobial interventions. *Food Control*, 26(2):610–613.

Planchon, S., Dargaignaratz, C., Levy, C., Ginies, C., Broussolle, V. & Carlin, F. (2011). spores of *Bacillus cereus* strain KBAB4 produced at 10°C and 30°C display variations in their properties. *Food Microbiology*, 28:291–297.

Pouillot, R., Albert, I., Cornu, M. & Denis, J. B. (2003). Estimation of uncertainty and variability in bacterial growth using bayesian inference. application to *Listeria monocytogenes*. *International Journal of Food Microbiology*, 81:87–104.

Pouillot, R., Lubran, M. B., Cates, S. C. & Dennis, S. (2010). Estimating parametric distributions of storage time and temperature of ready-to-eat foods for us households. *Journal of Food Protection*, 73(2):312–321.

Powers, E. M., Latt, T. G. & Brown, T. (1976). Incidence and levels of *Bacillus cereus* in processed spices. *Journal of Milk and Food Technology*, 39(10):668–670.

Prescott, S. C. & Underwood, W. L. (1897). Contributions to our knowledge of micro-organisms and sterilizing processes in the canning industries. *Science*, 6(152):800–802.

Quintavalla, S. & Parolari, G. (1993). Effects of temperature, a_w and pH on the growth of *Bacillus* cells and spores: a response surface methodology study. *International Journal of Food Microbiology*, 19(3):207–216.

Rajkovic, A. (2006). *Biological and immuno-molecular techniques for monitoring of Bacillus cereus emetic toxin and Staphylococcus aureus enterotoxin*. Ph.D. thesis, Faculty of Bioscience Engineering, Ghent University.

Rajkovic, A., Kljajic, M., Smigic, N., Devlieghere, F. & Uyttendaele, M. (Accepted). Toxin producing *Bacillus cereus* persist in food mainly in vegetative form. *International Journal of Food Microbiology*.

Rajkovic, A., Uyttendaele, M., Courtens, T. & Debevere, J. (2005). Antimicrobial effect of nisin and carvacrol and competition between *Bacillus cereus* and *Bacillus circulans* in vacuum-packed potato puree. *Food Microbiology*, 22(2-3):189–197.

Rajkovic, A., Uyttendaele, M., Courtens, T., Heyndrickx, M. & Debevere, J. (2006). Prevalence and characterisation of *Bacillus cereus* in vacuum packed potato puree. *International Journal of Food Science and Technology*, 41(8):878–884.

Rajkovic, A., Uyttendaele, M., Vermeulen, A., Andjelkovic, M., Fitz-James, I., in't Veld, P., Denon, Q., Verhe, R. & Debevere, J. (2008). Heat resistance of *Bacillus cereus* emetic toxin, cereulide. *Letters in Applied Microbiology*, 46(5):536–541.

Redmond, E. C. & Griffith, C. J. (2003). Consumer food handling in the home: A review of food safety studies. *Journal of Food Protection*, 66(1):130–161.

Reij, M. W., Den Aantrekker, E. D. & in Microbiology Task Force, I. E. R. A. (2004). Recontamination as a source of pathogens in processed foods. *International Journal of Food Microbiology*, 91(1):1–11.

Robinson, R., Batt, A. & D.P., P., editors (2000). *Encyclopedia of Food Microbiology*, volume 2. Academic Press.

Robinson, T. P., Ocio, M. J., Kaloti, A. & Mackey, B. M. (1998). The effect of the growth environment on the lag phase of *Listeria monocytogenes*. *International Journal of Food Microbiology*, 44(1-2):83–92.

Ross, T. & Dalgaard, P. (2004). Modeling microbial responses in food. In McKellar, R. C. & Lu, X., editors, *Modeling microbial responses in food*, chapter Secondary models, pp. 63–150. CRC Press, Boca Raton.

Rosso, L., Bajard, S., Flandrois, J. P., Lahellec, C., Fournaud, J. & Veit, P. (1996). Differential growth of *Listeria monocytogenes* at 4 and 8 degrees C: Consequences for the shelf life of chilled products. *Journal of Food Protection*, 59(9):944–949.

Rosso, L., Lobry, J., Bajard, S. & Flandrois, J. P. (1995). Convenient model to describe the combined effects of temperature and pH on microbial growth. *Applied and Environmental Microbiology*, 61(2):610–616.

Samapundo, S., Everaert, H., Wandutu, J. N., Rajkovic, A., Uyttendaele, M. & Devlieghere, F. (2011a). The influence of headspace and dissolved oxygen level on growth and haemolytic bl enterotoxin production of a psychrotolerant *Bacillus weihenstephanensis* isolate on potato based ready-to-eat food products. *Food Microbiology*, 28(2):298–304.

Samapundo, S., Heyndrickx, M., Xhaferi, R. & Devlieghere, F. (2011b). Incidence, diversity and toxin gene characteristics of *Bacillus cereus* group strains isolated from food products marketed in Belgium. *International Journal of Food Microbiology*, 150(1):34–41.

Samapundo, S., Heyndrickx, M., Xhaferi, R. & Devlieghere, F. (2011c). Validated empirical models describing the combined effect of water activity and pH on the heat resistance of spores of a psychrotolerant *Bacillus cereus* strain in broth and bechamel sauce. *Journal of Food Protection*, 74(10):1662–1669.

Sampers, I., Berkvens, D., Jacxsens, L., Ciocci, M. C., Dumoulin, A. & Uyttendaele, M. (2012). Survey of Belgian consumption patterns and consumer behaviour of poultry meat to provide insight in risk factors for campylobacteriosis. *Food Control*, 26(2):293–299.

Sanaei-Zadeh, H. (2012). Can *Bacillus cereus* food poisoning cause sudden death? *Journal of Clinical Microbiology*, 50(11):3816.

Sanchez-Salas, J.-L., Setlow, B., Zhang, P., Li, Y. & Setlow, P. (2011). Maturation of released spores is necessary for acquisition of full spore heat resistance during *Bacillus subtilis* sporulation. *Applied and Environmental Microbiology*, 77(19):6746–6754.

Scallan, E., Hoekstra, R. M., Angulo, F., Tauxe, R., Widdowson, M., Roy, S., Jonesn, J. & Griffin, P. M. (2011). Foodborne illness acquired in the United States - major pathogens. *Emerging Infectious Diseases*, 17(1):7–15.

Scott, V. N. & Bernard, D. (1985). the effect of lysozyme on the apparent heat-resistance of nonproteolytic type-b *Clostridium botulinum*. *Journal of Food Safety*, 7(3):145–154.

Sergeledis, D., Abrahim, A., Sarimvei, A., Panoulis, C., Karaioannoglou, P. & Genigeorgis, C. (1997). Temperature distribution and prevalence of *Listeria* spp. in domestic, retail and industrial refrigerators in greece. *International Journal of Food Microbiology*, 34:171–177.

Setlow, P. (2003). Spore germination. *Current Opinion in Microbiology*, 6(6):550–556.

Setlow, P. (2006). Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology*, 101:514–525.

Sinigaglia, M., Corbo, M. R., Altieri, C. & Massa, S. (2002). Response surface model for effects of temperature, water activity and pH on germination of *Bacillus cereus* spores. *Journal of Food Safety*, 22(2):121–133.

Smelt, J. P. P. M., Bos, A. P., Kort, R. & Brul, S. (2008). Modelling the effect of sub(lethal) heat treatment of *Bacillus subtilis* spores on germination rate and outgrowth to exponentially growing vegetative cells. *International Journal of Food Microbiology*, 128(1):34–40.

Smelt, J. P. P. M., Otten, G. & Bos, A. P. (2002). Modelling the effect of sublethal injury on the distribution of the lag times of individual cells of *Lactobacillus plantarum*. *International Journal of Food Microbiology*, 73:207–212.

Stringer, S. C., Webb, M. D. & Peck, M. W. (2011). Lag time variability in individual spores of *Clostridium botulinum*. *Food Microbiology*, 28:228–235.

Stumbo (1949). Considerations relating to evaluation of thermal processes in foods. *Food Technology*, 3:126–131.

Stumbo, C. R. (1948). Bacteriological considerations relating to process evaluation. *Food Technology*, 2:115–132.

Stumbo, C. R. (1965). *Thermobacteriology in food processing*. Academic Press, Ins. New York.

Takabe, F. & M., O. (1976). An autopsy case of food poisoning associated with *Bacillus cereus*. *Forensic Science*, 7(2):97–101.

Ter Beek, A., Hornstra, L. M., Pandey, L., Kallemeijn, W., Smelt, J. P. P. M., Manders, E. & Brul, S. (2011). Models of the behaviour of (thermally stressed) microbial spores in foods: Tools to study mechanisms of damage and repair. *Food Microbiology*, 28:678–684.

Terpstra, M., Steenbekkers, L., de Maertelaere, N. & Nijhuis, S. (2005). Food storage and disposal: consumer practices and knowledge. *British Food Journal*, 107(7):526–533.

Teyssou, R., Hance, P. & Buisson, Y. (1998). Les infections humaines à *Bacillus*. *Bulletin de la Societe Francaise de Microbiologie*, 13:137–144.

The Weather Channel (2012). Average daily temperature for Brussels, Belgium. Accessed on December 18, 2012. URL http://www.weather.com/weather/wxclimatology/daily/BEXX0005.

Thomas, L., Ingram, R., Bevis, H., Davies, C. E.A.and Milne & Delves-Broughton, J. (2001). Effective use of nisin to control *Bacillus* and *Clostridium* spoilage of a pasteurized mashed potato product. *Journal of Food Protection*, 65(10):1580–1585.

Thorsen, L., Budde, B., Henrichsen, L., Martinussen, T. & Jakobsen, M. (2009). Cereulide formation by *Bacillus weihenstephanensis* and mesophilic emetic *Bacillus cereus* at temperature abuse depends on pre-incubation conditions. *International Journal of Food Microbiology*, 134(1-2):133–139.

Tompkin, R. B. (2002). Control of *Listeria monocytogenes* in the food-processing environment. *Journal of Food Protection*, 65(4):709–725.

Turnbull, P. C. B., Frawley, D. A. & Bull, R. L. (2007). Heat activation/shock temperatures for *Bacillus anthracis* spores and the issue of spore plate counts versus true numbers of spores. *Journal of Microbiological Methods*, 68(2):353–357.

UK Department of Health (1994). *Guidelines for the safe production of heat preserved foods*. HMSO, London.

Unklesbay, N., Sneed, J. & Toma, R. (1998). College students' attitudes, practices, and knowledge of food safety. *Journal of Food Protection*, 61(9):1175–1180.

US FDA (1999). U.s. food temperature evaluation design and summary pages. Technical report, U.S. Food and Drug Administration. URL http://foodrisk.org/default/assets/File/Audits-FDA_temp_study.pdf.

US FDA (2003). Quantitative assessment of relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods. Technical report, Center for Food Safety and Applied Nutrition; U.S. Department of Agriculture, Food Safety and Inspection Service; Centers for Disease Control and Prevention. URL http://www.fda.gov/downloads/food/scienceresearch/researchareas/riskassessmentsafetyassessment/ucm197330.pdf.

Uyttendaele, M., Jacxsens, L., De Loy-Hendrickx, A., Devlieghere, F. & Debevere, J. (2010). *Microbiologische richtwaarden en wettelijke microbiologische criteria*. LFMFP-UGent, Ghent. ISBN: 978-90-5989-385-6.

Valero, M., Hernandez-Herrero, L. A., Fernandez, P. S. & Salmeron, M. C. (2002). Characterization of *Bacillus cereus* isolates from fresh vegetables and refrigerated minimally processed foods by biochemical and physiological tests. *Food Microbiology*, 19(5):491–499.

van Asselt, E. D. & Zwietering, M. H. (2006). A systematic approach to determine global thermal inactivation parameters for various food pathogens. *International Journal of Food Microbiology*, 107(1):73–82.

Van Boekel, M. (2002). On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *International Journal of Food Microbiology*, 74(1-2):139–159.

Van Boekel, M., Fogliano, V., Pellegrini, N., Stanton, C., Scholz, G., Lalljie, S., Somoza, V., Knorr, D., Jasti, P. R. & Eisenbrand, G. (2010). A review on the beneficial aspects of food processing. *Molecular Nutrition & Food Research*, 54(9):1215–1247.

Van Boxstael, S., Devlieghere, F., Berkvens, D., Vermeulen, A. & Uyttendaele, M. (Under Review). Understanding and attitude regarding the shelf life labels and dates on pre-packed food products by Belgian consumers. *Food Control*.

Van Der Voort, M. & Abee, T. (2009). Transcriptional regulation of metabolic pathways, alternative respiration and enterotoxin genes in anaerobic growth of *Bacillus cereus* atcc 14579. *Journal of Applied Microbiology*, 107(3):795–804.

Van Schothorst, M. (2004). *A simple guide to understanding and applying the hazard analysis critical control point concept.* Concise Monograph Series. ILSI Europe, Brussels, 3rd edition. ISBN 1-S7881179-1.

Verbeke, W., Frewer, L., Scholderer, J. & De Brabander, H. (2007). Why consumers behave as they do with respect to food safety and risk information. *Analytica Chimica Acta*, 586:2–7.

Vermeulen, A., Daelman, J., Van Steenkiste, J. & Devlieghere, F. (2012). Screening of different stress factors and development of growth/no growth models for *Zygosaccharomyces rouxii* in modified sabouraud medium, mimicking intermediate moisture foods (IMF). *Food Microbiology*, 32(2):389–396.

Vermeulen, A., Devlieghere, F., De Loy-Hendrickx, A. & Uyttendaele, M. (2011). Critical evaluation of the EU-technical guidance on shelf-life studies for *L. monocytogenes* on RTE-foods: A case study for smoked salmon. *International Journal of Food Microbiology*, 145(1):176–185.

Vermeulen, A., Gysemans, K. P. M., Bernaerts, K., Geeraerd, A. H., Van Impe, J. F., Debevere, J. & Devlieghere, F. (2007a). Influence of pH, water activity and acetic acid concentration on *Listeria monocytogenes* at 7 degrees C: Data collection for the development of a growth/no growth model. *International Journal of Food Microbiology*, 114(3):332–341.

Vermeulen, A., Smigic, N., Rajkovic, A., Gysemans, K., Bernaerts, K., Geeraerd, A., van Impe, J., Debevere, J. & Devlieghere, F. (2007b). Performance of a growth-no growth model for *Listeria monocytogenes* developed for mayonnaise-based salads: Influence of strain variability, food matrix, inoculation level, and presence of sorbic and benzoic acid. *Journal of Food Protection*, 70(9):2118–2126.

Voedingscentrum (1999). Hygiene in de privéhuishouding, rapport nr 10160. Technical report, Research International.

Warth, A. (1980). Heat stability of *Bacillus cereus* enzymes within spores and in extracts. *Journal of Bacteriology*, 143:27–34.

WHO/FAO (2004). Risk assessment of *Listeria monocytogenes* in ready-to-eat foods, no. 5. Technical report, World Health Organisation/Food and Agriculture Organization.

Wijnands, L. M., Dufrenne, J. B., Rombouts, F. M., In't Veld, P. H. & Van Leusden, F. M. (2006). Prevalence of potentially pathogenic *Bacillus cereus* in food commodities in the netherlands. *Journal of Food Protection*, 69(11):2587–2594.

Wijtzes, T., van't Riet, K., Huis in't Veld, J. H. J. & Zwietering, M. H. (1998). A decision support system for the prediction of microbial food safety and food quality. *International Journal of Food Microbiology*, 42(1-2):79–90.

Wollstonecroft, M. M., Ellis, P. R., Hillman, G. C., Fuller, D. Q. & Butterworth, P. J. (2012). A calorie is not necessarily a calorie: Technical choice, nutrient bioaccessibility, and interspecies differences of edible plants. *Proceedings of the National Academy of Sciences of the United States of America*, 109(17):E991–E991.

Wong, H. C. & Chen, Y. L. (1988). Effects of lactic-acid bacteria and organic-acids on growth and germination of *Bacillus cereus*. *Applied and Environmental Microbiology*, 54(9):2179–2184.

World Trade Organization (1995). Agreement on the application of sanitary and phytosanitary measures. Availble from `http://www.wto.org/english/docs_e/legal_e/15-sps.pdf`.

Wrangham, R. & Conklin-Brittain, N. (2003). Cooking as a biological trait. *Comparative Biochemistry and Physiology a Molecular and Integrative Physiology*, 136(1):35–46.

Yiannas, F. (2009). *Food Safety Culture: Creating a Behavior-Based Food Safety Management System*. Food Microbiology and Food Safety Series. Springer. ISBN: 978-0-387-72867-4.

Zwietering, M. H. (2002). Quantification of microbial quality and safety in minimally processed foods. *International Dairy Journal*, 12(2-3):263–271.

Zwietering, M. H. (2005). Practical considerations on food safety objectives. *Food Control*, 16(9):817–823.

Zwietering, M. H. (2009). Quantitative risk assessment: Is more complex always better? simple is not stupid and complex is not always more correct. *International Journal of Food Microbiology*, 134(1-2):57–62.

Zwietering, M. H., Cuppers, H. G. A. M., Dewit, J. C. & Vantriet, K. (1994). Evaluation of data transformations and validation of a model for the effect of temperature on bacterial-growth. *Applied and Environmental Microbiology*, 60(1):195–203.

Acknowledgements

This study was funded by the **Federal Public Service Health, Food Chain Safety and Environment** (Contract RT 09/01 MICRORISK).

The author wishes to thank,

- the **Federal Public Service Health, Food Chain Safety and environment**, for their financial support of this project.
- the **Scientific Research Committee of the Faculty of Bioscience Engineering** at Ghent University for enabling a stay in Nantes, which led to the development of several models and the final exposure assessment model.
- the **REPFED companies** and the **Belgian Ready Meal Association** (BREMA) for the fruitful cooperation, for providing product samples, microbiological data and their expert opinion.
- **Flanders Food**, for allowing us to use their *B. cereus* strains.
- **Chris Drieskens** for writing the excel application for data processing.
- **Elien Verguldt** for her continuous commitment to this research project and for the tremendous amount of lab work she did.

Curriculum vitae

Jeff Daelman was born in Ghent on the 30th of december 1986. In 2004 he finished high school, and five years later he graduated as a Master of Science in Bioscience Engineering: Food Science and Nutrition. During his years at university, he was active in the students' association of the faculty.

He did his master thesis, concerning the microbial stability of intermediate moisture foods, at the Lab for Food Microbiology and Food Preservation (faculty of Bioscience Engineering). In september of 2009, he began working as a PhD researcher at the same lab. For his research project, he cooperated with several Belgian REPFED producers. In addition, part of the research was performed at the Unité de recherche Sécurité des Aliments et Microbiologie (Oniris, Nantes, France) under the guidance of dr. Jeanne-Marie Membré. In the framework of his research, several REPFED processing plants were sampled, a lag model was developed for heat-treated *B. cereus* spores, a survey was conducted into consumer behaviour and several risk mitigation strategies were compared. He completed this PhD under the guidance of his promotors at Ghent University: prof. dr. ir. Mieke Uyttendaele, prof. dr. ir. Frank Devlieghere and prof. dr. ir. Liesbeth Jacxsens.

During his research, he participated in several national and international conferences and published in international journals. In addition, he guided several master thesis students and assisted in the practical sessions of the course 'food microbiology'. In the framework of the project, he also answered more specific questions of the participating companies. During his PhD he was active in the alumni association of the faculty.

Curriculum vitae

Jeff Daelman werd geboren op 30 december 1986 te Gent. In 2004 behaalde hij het diploma wiskunde-wetenschappen aan het Sint-Vincentius a Paulo instituut te Gijzegem, vijf jaar later promoveerde hij tot Bio-ingenieur in de levensmiddelenwetenschappen en voeding. Tijdens zijn studententijd was hij tevens actief in de studentenvereniging van de faculteit.

Zijn masterproef, omtrent de microbiële stabiliteit van zoetwaren, deed hij aan het Labo voor Levensmiddelenmicrobiologie en -conservering (Faculteit Bio-ingenieurswetenschappen). In september 2009, startte hij als doctoraatsbursaal op hetzelfde labo. Voor zijn onderzoeksproject werkte hij samen met verschillende Belgische producenten van kant-en-klare maaltijden. Daarnaast werd een deel van het onderzoek uitgevoerd aan het Unité de recherche Sécurité des Aliments et Microbiologie (Oniris, Nantes, Frankrijk) onder begeleiding van dr. Jeanne-Marie Membré. In het kader van het project werden verschillende productievestigingen van kant-en-klare maaltijden bemonsterd, werd een lag-model voor hittebehandelde *B. cereus* sporen ontwikkeld, werd gepeild naar consumentengedrag en werden verscheidene strategieën voor risicobeheersing vergeleken. Hij werkte aan dit doctoraat onder leiding van zijn promoteren, prof. dr. ir. Mieke Uyttendaele, prof. dr. ir. Frank Devlieghere en prof. dr. ir. Liesbeth Jacxsens, allen verbonden aan de UGent.

Tijdens zijn onderzoek, nam hij deel aan verscheidene nationale en internationale conferenties en publiceerde hij artikels in internationale tijdschriften. Verder begeleidde hij verscheidene thesisstudenten en werkte hij mee aan de practica voor het vak 'levensmiddelenmicrobiologie' in de opleiding Bio-ingenieur. In het kader van de samenwerking met de sector van de kant-en-klare maaltijden behandelde hij ook specifieke vragen van de deelnemende bedrijven. Daarnaast was hij actief in de alumnivereniging van de faculteit.

List of scientific publications

Publications in international peer-reviewed journals

- Vermeulen, A., Daelman, J., Van Steenkiste, J. & Devlieghere, F. (2012). Screening of different stress factors and development of growth/no growth models for *Zygosaccharomyces rouxii* in modified sabouraud medium, mimicking intermediate moisture foods (IMF). *Food Microbiology*, 32(2):389–396

- Lahou, E., Jacxsens, L., Daelman, J., Van Landeghem, F. & Uyttendaele, M. (2012). Microbiological performance of a food safety management system in a food service operation. *Journal of Food Protection*, 75(4):706–716

- Daelman, J., Jacxsens, L., Devlieghere, F. & Uyttendaele, M. (2013b). Microbial safety and quality of various types of cooked chilled foods. *Food Control*, 30(2):510–517

- Daelman, J., Jacxsens, L., Lahou, E., Devlieghere, F. & Uyttendaele, M. (2013c). Assessment of the microbial safety and quality of cooked chilled foods and their production process. *International Journal of Food Microbiology*, 160(3):193–200

- Daelman, J., Vermeulen, A., Willemyns, T., Ongenaert, R., Jacxsens, L., Uyttendaele, M. & Devlieghere, F. (2013f). Growth no growth models for heat-treated psychrotrophic *Bacillus cereus* spores under cold storage. *International Journal of Food Microbiology*, 161(1):7–15

- Daelman, J., Aditya, S., Vermeulen, A., Uyttendaele, M., Devlieghere, F. & Membré, J. M. (2013a). Development of a time-to-detect growth model for heat-treated *Bacillus cereus* spores. *International Journal of Food Microbiology*, 165(3):231–240

- Daelman, J., Jacxsens, L., Membré, J. M., Sas, B., Devlieghere, F. & Uyttendaele, M. (2013d). Behaviour of Belgian consumers, related to the consumption, storage and preparation of cooked chilled foods. *Food Control*, 34(2):681–690

- Daelman, J., Membré, J. M., Jacxsens, L., Vermeulen, A., Devlieghere, F. & Uyttendaele, M. (2013e). A Quantitative Microbiological Exposure Assessment model for *Bacillus cereus* in REPFEDs. *International Journal of Food Microbiology*, 166(23):433–449

Conferences with oral presentation

- Daelman, J., Jacxsens, L., Lahou, E. & Uyttendaele, M. (2011). A microbial assessment scheme of the cooked chilled food production. *16th PhD symposium on Applied Biological Sciences*, Ghent, Belgium.

- Daelman J., & Guido Bresseleers, Case study: Veiligheid van gepasteuriseerde kant-en-klare maaltijden- FOD Volksgezondheid MicroRisk project, *16th Conference on Food Microbiology (Belgian Society of Food Microbiology)*, Brussels, Belgium.

- Daelman, J., Jacxsens, L., Vermeulen, A., Uyttendaele, M. & Devlieghere, F. (2011). Growth/no-growth models for heat-treated spores of psychrotrophic *Bacillus cereus* strains. *7th International conference on Predictive Modelling of Food Quality and Safety*, Dublin, Ireland.

- Daelman, J., Membré, J. M., Uyttendaele, M. & Devlieghere, F. (2012). A bayesian model for *Bacillus cereus* contamination in raw materials used for cooked chilled food production. *Belgian-Dutch Conference on Machine Learning (Benelearn)*, Ghent, Belgium.

- Daelman, J., Membré, J. M., Uyttendaele, M. & Devlieghere, F. (2012). A bayesian model for *Bacillus cereus* contamination in raw materials used for REPFEDs production. *2012 IAFP European Symposium*, Warsaw, Poland.

- Daelman, J., Membré, J. M., Jacxsens, L., Devlieghere, F. & Uyttendaele, M. (2012). A quantitative microbiological risk assessment of *Bacillus cereus* in cooked chilled foods. *23rd internal ICFMH Symposium, FoodMicro 2012*, Istanbul, Turkey.

- Daelman, J., Membré, J. M., Jacxsens, L., Devlieghere, F. & Uyttendaele, M. (2013). Consumer habits and their impact on the food safety of cooked chilled foods - results of a survey in Belgium. *2013 IAFP European Symposium*, Marseille, France.

- Daelman, J., Membré, J. M., Jacxsens, L., Uyttendaele, M. & Devlieghere, F. (2013). A quantitative exposure assessment of *Bacillus cereus* in cooked chilled foods. *8th International conference on Predictive Modelling of Food Quality and Safety*, Paris, France.

- Vermeulen, A., Deschuyffeleer, N., Daelman, J., Eeckhout, M. & Devlieghere, F. (2013). Combining growth/no growth models for osmophilic yeast and xerophilic moulds to predict the stability of IMF *8th International conference on Predictive Modelling of Food Quality and Safety*, Paris, France.

Conferences with poster presentation

- Daelman, J., Jacxsens, L., Geeraerd, A., Van Impe, J., Devlieghere, F. & Uyttendaele, M. (2010). Minimally heat-processed foods: Quantitative Microbiological Risk Assessment vs. traditional safe harbours. *22rd internal ICFMH Symposium, FoodMicro 2010*, Copenhagen, Denmark.

- Daelman, J., Jacxsens, L., Devlieghere, F. & Uyttendaele, M. (2011). Microbial risk profiling of cooked chilled foods, *2011 IAFP European symposium*, Ede, The Netherlands.

- Daelman, J., Jacxsens, L., Lahou, E., Devlieghere, F. & Uyttendaele, M. (2011). A microbial assessment scheme for the cooked chilled food production process, *2011 IAFP European symposium*, Ede, The Netherlands.

- Daelman, J., Jacxsens, L., Devlieghere, F. & Uyttendaele, M. (2011). A framework for validation of the microbial safety of cooked chilled foods, *2011 IAFP annual meeting*, Milwaukee (WI), USA.

- Daelman, J., Membré, J. M., Jacxsens, L., Devlieghere, F. & Uyttendaele, M. (2012). A bayesian model for *Bacillus cereus* contamination in raw materials used in cooked chilled foods.. *23rd internal ICFMH Symposium, FoodMicro 2012*, Istanbul, Turkey.

- Daelman, J., Vermeulen, A., Uyttendaele, M., Devlieghere, F., & Membré, J. M. (2013) Modelling the lag time of *Bacillus cereus* spores as a function of thermal heat-treatment intensity, product formulation (pH and a_w) and chilled storage conditions. *8th International conference on Predictive Modelling of Food Quality and Safety*, Paris, France.